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Abscisic acid inhibits germination of mature *Arabidopsis* seeds by limiting the availability of energy and nutrients

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Abstract. The addition of abscisic acid (ABA) to mature non-dormant seeds inhibits their germination. This effect of ABA might be related to its natural function as an endogenous inhibitor of precocious germination during seed formation. In this work, we studied how ABA affects the germination of mature seeds and the growth of nascent seedlings of *Arabidopsis thaliana* (L.) Heynh. Our findings were as follows: (i) inhibition by ABA was gradual, dose-dependent, and did not disappear after germination; (ii) inhibition of germination was relieved by the addition of metabolizable sugars or amino acids to the plating media; (iii) the effect of sugars and amino acids was cooperative, indicating that these two groups of metabolites relieve different deficiencies; (iv) ABA caused appreciable alterations in energy and nitrogen metabolism; and (v) ABA prevented the degradation of the seed storage proteins. In summary, ABA appears to inhibit seed germination by restricting the availability of energy and metabolites. This mechanism seems consistent with other known effects of ABA.

Key words: Abscisic acid – *Arabidopsis* – Carbon metabolism – Germination – Nitrogen metabolism – Storage protein

Introduction

The phytohormone abscisic acid (ABA) is universally present in the developing seeds of higher plants, where it participates in a wide spectrum of physiological, biochemical, and molecular processes (Quatrano 1986; Black 1991). Three much-studied classes of ABA-insensitive *Arabidopsis* mutants (*abi1*, *abi2*, *abi3*) were selected for being able to germinate in the presence of 10 μM exogenous ABA (Koornneef et al. 1984). Although their functions overlap, *abi3* is most important for seed development while *abi1* and *abi2* are more relevant in the mature plant (Finkelstein and Somerville 1990).

The *ABI3* locus has been cloned, and was found to be a homologue of the maize *VP1* gene (Giraudat et al. 1992). *VP1* is known to be a transcriptional activator (McCarty et al. 1991) and repressor (Hoecker et al. 1995), and is required for the response to exogenous ABA. Null *abi3* mutants, or leaky *abi3* mutants combined with ABA deficiency, have shown that ABA is required to keep the seeds in an embryonic program and to prevent precocious germination (Nambara et al. 1992; Ooms et al. 1993).

Vp1 mutants in maize show incomplete accumulation of storage proteins (Rivin and Grudt 1991). So do the *Arabidopsis* mutants in *abi3* (Finkelstein and Somerville 1990; Rivin and Grudt 1991; Finkelstein 1993) which also show deficiency of other reserve molecules, such as eicosenoic acid, the major component in storage lipids (Finkelstein and Somerville 1990).

It has been implied that ABA is involved in the acquisition of desiccation tolerance during late maturation, supposedly through the induction of late embryogenesis-abundant (LEA) proteins. Accordingly, the *abi3* mutant of *Arabidopsis* is desiccation intolerant and fails to induce at least one LEA protein that was tested (Ooms et al. 1993). Desiccation, besides guaranteeing a stable quiescent state, might be required for the switching from an embryonic to a germinative program (Kermode et al. 1986). Germination, which seems to be the reversal of seed maturation, implies rapid rehydration, reactivation of metabolism and meristematic growth, and mobilization of the accumulated reserves (Black 1991). Some of these processes, and germination as a whole, can be inhibited by the application of ABA.

Abbreviations: A = MM + (±) –ABA; ABA = abscisic acid; AG = A + glucose; AGP = AG + peptone; AM = A + maltose; AP = A + peptone; AS = A + sucrose; DAP = days after plating; G = MM + glucose; GP = G + peptone; M = MM + maltose; MM = minimal medium; P = MM + peptone; S = MM + sucrose

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The in-vivo relevance of this inhibition has been questioned since, by the time the seed is mature, endogenous ABA has usually dropped to very low levels (Black 1991).

One of the best-studied effects of ABA during germination is the blocking (in opposition to gibberellin) of the induction of α-amylase in the aleurone of cereals (Higgins et al. 1982; Oishi and Bewley 1990). Surprisingly, ABA inhibits transcription from the α-amylase promoter even in the absence of the *VP1* protein, while *VP1* represses the α-amylase promoter even when ABA is very low (Hoecker et al. 1995). Other examples of ABA being related to reserve mobilization include the inhibition of α-amylase in *Phaseolus vulgaris* (Van Onckelen et al. 1980), the reduction in the levels of isocitrate lyase mRNA (Dommes and Northcote 1985), and the inhibition of mannanase activity (Malek and Bewley 1991).

In this work we explored whether or not the inhibition of germination of mature *Arabidopsis* seeds by ABA is related to the inhibition of reserve mobilization. We found that the addition of sugars and amino acids allowed the seeds to germinate in otherwise inhibitory concentrations of ABA. In relation to this, we also found that applied ABA altered the pools of available nitrogen and energy. Finally, we demonstrate that this hormone prevents the degradation of the seed storage proteins.

Materials and methods

Chemicals and biological material. Most chemicals were from Sigma Chemical Co. (St. Louis, Mo., USA). Bacto-agar and peptone were from DIFCO Laboratories (Detroit, Mch., USA). *Arabidopsis thaliana* ecotype Columbia seeds were from our stock and had been stored at 4 °C for at least one month and did not show dormancy.

Growth conditions. Sterilized seeds were plated in the indicated media and incubated at 22 °C with 16:8 h light:dark periods. Plating media were minimal (MM; Haughn and Somerville 1986) with 0.7% agar plus additions, as required: 10 μM (±)-ABA; 0.5% (w/v) glucose, 0.5% (w/v) sucrose; 0.5% (w/v) mannitol; 0.3% (w/v) peptone. Germination was quantified by radicle emergence from triplicates with 100 seeds each.

Protein isolation and analysis. Total proteins were isolated by a phenol extraction method (Hurkman and Tanaka 1986) and analyzed by SDS-PAGE (Laemmli 1970). Protein was quantified by the BIO-RAD protein assay (BIO-RAD Laboratories, Hercules, Calif., USA). The seed storage proteins were detected with 0.1% Coomassie Brilliant Blue. In-vivo protein labeling was carried out by incubating the seeds for 24 h in 0.3 ml of the required medium containing 394 $\mu Bq \cdot ml^{-1}$ of [^{35}S Met + Cys]-Trans-label (ICN Radiochemicals, Costa Mesa, Calif., USA). Radioactive proteins were detected by fluorography on XAR-5 X-ray films (Eastman Kodak Co., Rochester, N.Y., USA).

Extraction and quantification of ABA. Abscisic acid was extracted according to (Peña-Cortes et al. 1989) and quantified with the Phytodetek-ABA kit (Idetek, San Bruno, Calif., USA) which utilizes a monoclonal antibody specific to (+)-ABA, and is sensitive in the range of 0.02–5.0 pM.

Quantification of reducing sugars and ammonia. After harvesting, 100 seeds were homogenized in 100 μl of 30 mM HCl and centrifuged 10 min at 6000 · *g*. Reducing sugars in the supernatants were determined by the method of Summer (Summer and Howell 1935). They are referred to a glucose standard curve and reported in nanomoles of glucose equivalents. Ammonia in the supernatants was quantified by the method of Kaplan (Kaplan 1965) with some modifications: 4 μl of supernatant was mixed with 150 μl of solution A (0.1 M phenol, 0.17 mM sodium nitroferricyanide). Then, 150 μl of solution B (0.155 M NaOH, 11 mM sodium hypochlorite) was added. After incubation at room temperature for 45 min, optical density at 575 nm was determined.

Amino acid analysis. The method described by Slocum was followed (Slocum and Cummings 1991). Homogenates from 100 seeds were deproteinized by precipitation with 10% sulfosalicylic acid. After neutralization, concentration, and further clarification, one-fifth of the sample was analyzed with a Beckman Amino Acid Analyzer (Model 6300) provided with a ion-exchange column (Beckman 338051) with ninhydrin detection.

Results

Abscisic acid causes a dose-dependent delay in germination which can be alleviated by addition of sugars and amino acids. Arabidopsis seeds were plated in MM with different concentrations of (±)-ABA and germination was quantified at 3, 7 and 14 days after plating (DAP; Table 1). At low concentrations, ABA retarded germination but did not prevent it. The retardation increased with concentration and also affected post-germinative

Table 1. Effect of increasing the concentration of (±)-ABA on the percentage germination of *Arabidopsis* seeds on different media. The indicated media were prepared with the ABA concentration indicated on top of each column. Seeds were sown in these media and germination was assessed at the indicated days as radicle emergence. Data are the average of triplicates. MM, minimal medium; M, MM + maltose; S, MM + Sucrose; G, MM + glucose; P, MM + peptone; GP, G + peptone

Medium	Day 3 after sowing					Day 7 after sowing					Day 14 after sowing				
	ABA concentration (μM)														
	0	0.3	1	10	30	0	0.3	1	10	30	0	0.3	1	10	30
MM	99	57	7	0	0	99	98	71	0	0	99	99	92	0	0
M	99	55	4	0	0	100	98	57	0	0	100	99	83	0	0
S	99	69	23	0	0	100	98	81	8	7	100	100	98	30	24
G	99	89	46	10	0	100	100	94	36	19	100	99	99	97	87
P	99	73	49	9	0	100	100	97	11	5	100	100	99	20	10
GP	99	98	60	14	4	100	99	98	89	68	100	100	99	99	98

Table 2. Effect of increasing the concentrations of sugars and peptone on the percentage germination of *Arabidopsis* seeds on MM medium alone or with (±)-ABA. The concentration of the required supplement (either manitol, sucrose, glucose, or peptone) is shown on the top of each column. AM, AS, AG, and AP media additionally contained 10 μM (±)-ABA. Germination was assessed at the indicated days as radicle emergence. Data are the average of triplicates

Medium	Day 3 after sowing						Day 7 after sowing						Day 14 after sowing					
	Concentration (% w/v)																	
	0	0.5	1	5	10	20	0	0.5	1	5	10	20	0	0.5	1	5	10	20
M	99	100	100	95	0	0	100	100	100	100	79	0	100	100	100	100	94	0
S	99	100	100	95	69	0	100	100	100	100	100	4	100	100	100	100	100	10
G	99	100	100	97	0	0	100	100	100	100	55	0	100	100	100	100	88	0
P	99	93	89	0	0	0	100	100	97	24	0	0	100	100	96	37	0	0
AM	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AS	0	0	7	18	0	0	0	11	11	38	20	0	0	29	47	66	59	0
AG	0	14	16	4	0	0	0	29	96	60	44	0	0	100	99	98	75	0
AP	0	12	2	0	0	0	0	16	8	0	0	0	0	18	10	3	0	0

growth. We did not find a threshold for this effect. At concentrations of 10 μM (±)-ABA, or higher, germination was not observed, but this can be considered as extreme retardation. These results suggest that ABA, rather than preventing germination directly, constrains some of the processes that lead to it. Reserve utilization could be such a process. When sucrose or glucose was provided, germination took place even when 30 μM (±)-ABA was present (Table 1, rows 3 and 4, respectively). Mannitol, a non-metabolizable sugar, was ineffective (Table 1, row 2). Interestingly, peptone, which is a pool of amino acids, caused similar relief (Table 1, row 5). The effect of amino acids was synergetic to that of glucose (compare rows 5 and 6 to row 7 in Table 1). For example, by day 7, germination in 10 μM (±)-ABA was 37% if glucose was present, 11% if peptone was, and as high as 89% if both were present. Thus, it can be concluded that amino acids are not just being used as energy, and that they and sugars counteract different deficiencies caused by ABA.

The effects of glucose, sucrose and peptone were further investigated. We found that 1, 5, and 0.5% were, respectively, their optimal concentrations for promoting germination in the presence of ABA (Table 2, rows 6–8). Higher concentrations actually produced a smaller effect. This could be explained because at high concentrations (10% or above) these nutrients inhibited germination by themselves (Table 2, rows 2–4). A similar inhibition was observed with mannitol, suggesting an osmotic effect (Table 2, row 1). A combination of 0.5% glucose and 0.3% peptone was chosen as standard for most experiments.

The endogenous ABA content, and the ability to detect it, are maintained in the presence of sugars and amino acids. To test if sugars or amino acids prevent ABA uptake, (+)-ABA content was assayed at 7 DAP in different media (Fig. 1). As expected, a lower ABA content was found in the seeds plated in MM alone (2 pg · mg^{-1} FW; Fig. 1, MM), than in MM with (±)-ABA (12 pg · mg^{-1} FW; Fig. 1, A). Those values were not significantly altered when glucose or peptone was included (Fig. 1, G, P, AG and AP). We conclude that

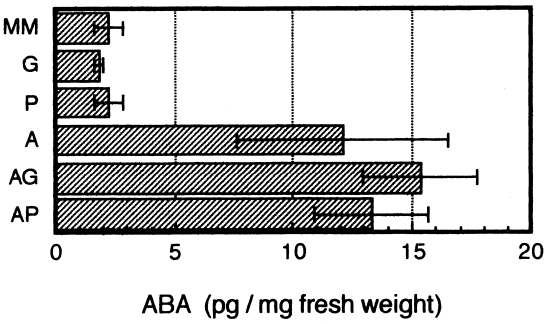

Fig. 1. Endogenous (+)-ABA content of *Arabidopsis* seeds under different treatments. Seven DAP in the indicated media, seeds were harvested and ABA content was determined. Each value is the mean ± SD of three independent measurements. Media abbreviations refer to minimal medium either alone (*MM*) or with supplements (*G*, glucose; *P*, peptone; *A*, ABA; *AG*, ABA + glucose; *AP*, ABA + glucose)

these metabolites do not favor germination by altering the intake or degradation of ABA.

To demonstrate that sugars and amino acids do not prevent the detection of ABA, we first searched for a biochemical marker of the seed's response to ABA. With this aim, we first investigated if ABA alters the protein-synthesis profile of the seed. Then, we investigated if these nutrients interfere with the ABA-specific alterations of the profile. Figure 2 shows the proteins synthesized by *Arabidopsis* seeds from 24 to 48 h after being plated in MM containing different additions. Several radiolabeled proteins were specifically induced by ABA (Fig. 2, lane A). The profile was not altered by the presence of glucose and peptone, indicating that these nutrients do not prevent the detection of ABA by the seed (Fig. 2, lane AGP). It is worth noting that in AGP the ABA-induced peptides still account for most of the protein synthesis activity, even if germination is underway due to the presence of the nutrients.

Abscisic acid alters the seed's carbon and nitrogen metabolism. To assess the effect of ABA on the seed's

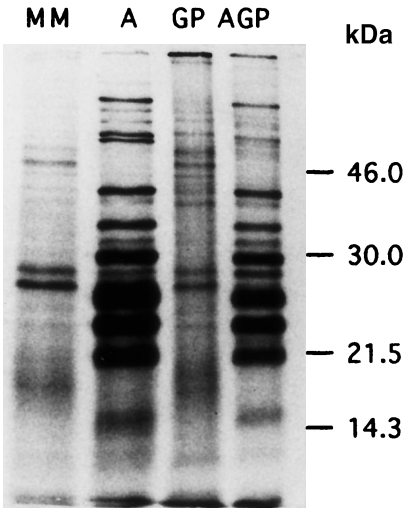

Fig. 2. Proteins synthesized de novo by *Arabidopsis* seeds planted in different media. The figure shows the fluorograph of an SDS-PAGE gel of in-vivo-labeled proteins. Labeling took place from 24 to 48 h post imbibition. *AGP*, minimal medium supplemented with ABA, glucose and peptone; other media abbreviations as in Fig. 1

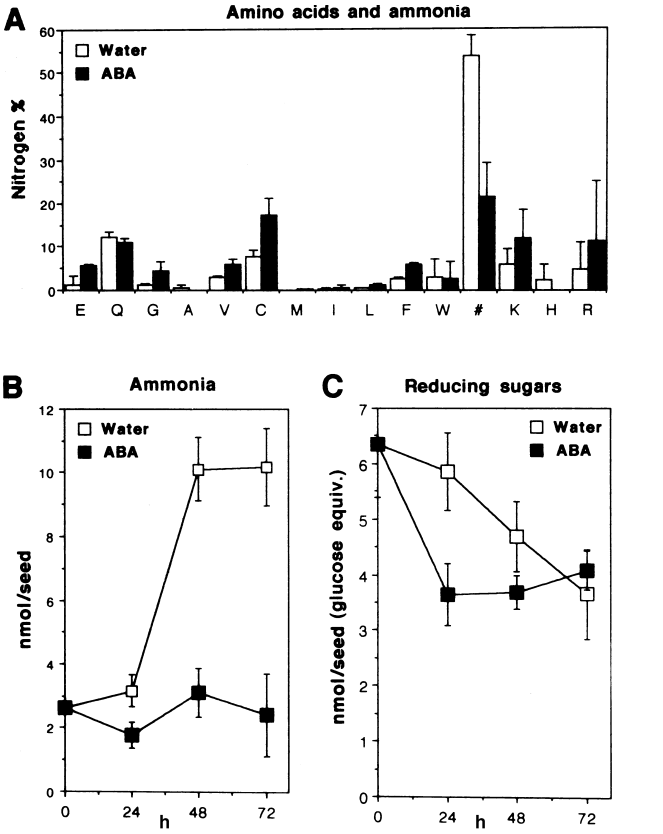

Fig. 3A–C. Metabolic indicators of the effect of ABA. Data refer to *Arabidopsis* seeds imbibed either in water or in 10 μM (±)-ABA. **A** Percentage of nitrogen contained as ammonia or amino acids in the seeds 48 h after plating. Amino acids are indicated by their standard single-letter codes, ammonia by a # symbol. The missing amino acids were not identified reproducibly. Values are means + SD of duplicates. **B** Content of ammonia at different times after plating. Ammonia was assayed colorimetrically. Amounts are reported in nmol per seed. Values are means ± SD of triplicates. **C** Content of reducing sugars at different times after plating. Amounts are reported per seed and expressed in nmol of glucose equivalents. Values are means ± SD of triplicates

amino acid pools, seeds were imbibed for 48 h either in water, or in 10 μM (±)-ABA, and their amino acid and ammonia contents were analyzed (Fig. 3A). The most striking, and most reliable, difference observed was for ammonia. Ammonia represented over 50% of all available nitrogen in the control seeds, but only half of that value in the ABA-treated seeds. Some amino acids also varied, especially glycine, glutamic acid, cysteine, arginine and lysine, but the relevance of this remains unclear. The strong effect of ABA on the ammonia pool was confirmed by quantifying ammonia colorimetrically at different times during germination. As shown in Fig. 3B, ammonia increased sharply, and over five-fold, between 24 and 48 h in the control seeds. In contrast, in the ABA-treated seeds, the ammonia content failed to rise during the 72 h following imbibition. Thus, ABA-imbibed seeds have a relative nitrogen deficiency. However, since their ammonia content remains at the initial level, it is possible that ABA causes this indirectly.

To obtain some indication of how ABA affects carbon metabolism, we assayed reducing sugars at different times after imbibition in water, or in 10 μM (±)-ABA (Fig. 3C). In the presence of ABA the content of reducing sugars in the seed halved within the first 24 h, and remained at that level thereafter. The behavior was markedly different in the control seeds where reducing sugars decreased gradually throughout the 72 h of germination. This result indicates that ABA can actively alter the seed's carbon metabolism.

Abscisic acid prevents the degradation of seed proteins. Figure 4A shows the fate of the main seed proteins during germination. At day 0, the characteristic 12S acidic and basic *Arabidopsis* storage proteins are clearly seen (Heath et al. 1986; Koornneef et al. 1989; Nambara et al. 1992). In the absence of ABA (GP) the degradation of these proteins has commenced by day 2, is extensive by day 3, and is complete before day 11. In contrast, in the presence of ABA (AGP) these proteins remain even at day 20, notwithstanding that the seeds have already become small plantlets (the average germination day is 7). In the experiment shown in Fig. 4B seeds from AG, AP and AGP media were hand-selected to ensure that they were all germinated, thus eliminating any possible noise from the few non-germinated seeds that could remain. As can be seen, the storage proteins were preserved in all conditions where ABA was present (A, AG, AP, and AGP), in sharp contrast to those without ABA (MM, G, and P). It should be noted that neither glucose nor peptone stabilized the storage proteins. Thus, a specific effect of ABA is the prevention of the degradation of the seed storage proteins.

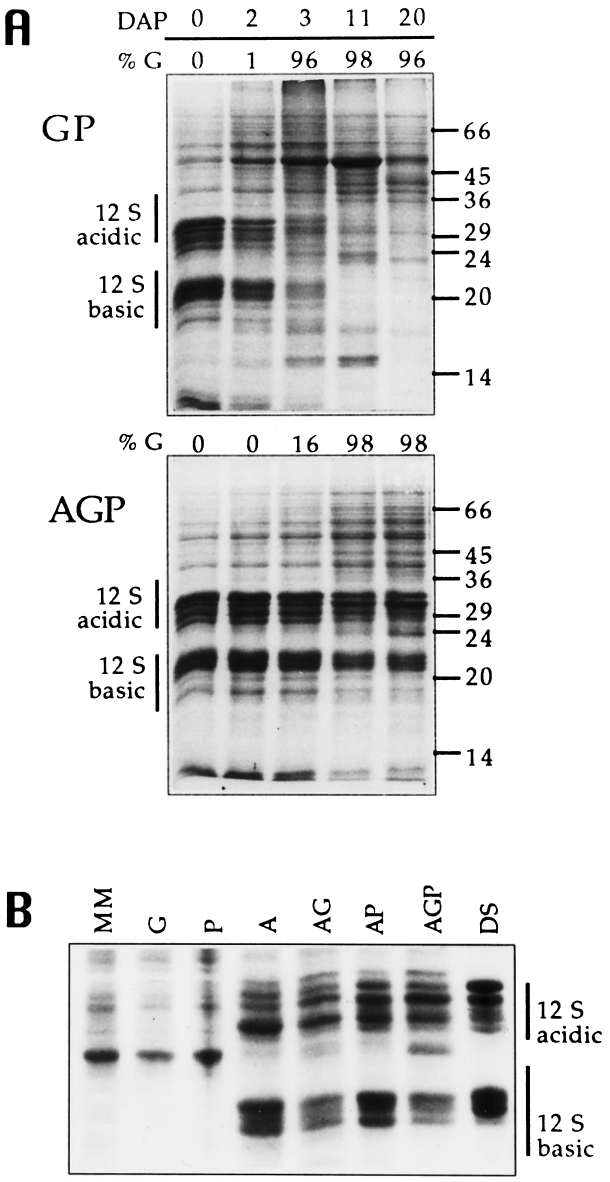

Fig. 4A,B. Effect of ABA on the fate of *Arabidopsis* storage proteins. **A** SDS-PAGE of total protein extracts obtained at different DAP either in GP or in AGP. The percentage germination of the corresponding seeds is shown above each lane (*%G*). The *Arabidopsis* storage-protein clusters are indicated (*12S acidic* and *12S basic*). **B** SDS-PAGE of total protein extracts obtained from *Arabidopsis* 11 DAP in different media. Dry seed proteins (*DS*) are included as a standard. Media abbreviations are as in Figs. 1 and 2

Discussion

Overall, our data suggest that ABA inhibits germination by causing a deficit of energy and building blocks. The best evidence is that inhibition is alleviated simply by providing sugars and amino acids to the seeds. The criticism that these additions could act as signals is unlikely because their optimal concentrations are too high and because they do not restore a normal germination rate, nor reinstate storage-protein degradation, as would be expected if these nutrients were pro-germinative signals overriding ABA as an anti-germinative signal.

In a study of the prevention by ABA of the precocious germination of *Pisum sativum* embryos in culture (Barratt et al. 1989), it was found that in a medium with 2% sucrose, ABA did not completely inhibit germination, but in 5% sucrose it did. This can be explained if, similarly to our findings with mature seeds, in immature embryos low concentrations of sucrose counteract ABA as a preventer of germination, while higher concentrations cause an inhibition of their own. This would suggest that ABA prevents germination by the same mechanism in both mature and immature seeds.

We found a steady decrease of reducing sugars in water-germinated seeds and a more abrupt one in ABA-imbibed seeds. We believe the former case is due to catabolic consumption of the sugars, while the later might be due to their polymerization. This interpretation agrees with the finding that, at the end of maturation, ABA-insensitive *Arabidopsis* seeds have higher than wild-type levels of several sugars and show an elevated ratio of mono- to oligosaccharides (Ooms et al. 1993).

We have shown that sugars and amino acids do not change the concentration of ABA in the seeds. We have also argued that they do not interfere with its detection since they do not prevent the induction of the ABA-specific protein synthesis profile. We would like to note that, although the profile served us as markers of the action of ABA, we do not know what its proteins are. A similar profile is induced by ABA in young plants, up to day 14, and in developing siliques (data not shown). The abundance of these proteins and their pattern of expression suggest that many of them could be late embryogenesis-abundant proteins.

We have shown that ABA prevents the degradation of storage proteins. Where do amino acids for germination come from in the AG condition? It could be that ABA causes an incomplete amino acid depletion, or that amino acid synthesis is facilitated by there being plenty of both inorganic nitrogen and an energy source, or that some turnover of macromolecules is taking place.

Our findings add to previous results showing that ABA seems to govern over the reserves usage/accumulation dichotomy. In maize, ABA is important in preventing precocious hydrolysis of storage compounds accumulating in the endosperm (Hoecker et al. 1995) and promotes the synthesis of lipid-body protein L3, which, interestingly, can also be dramatically re-induced in mature seeds if imbibed in ABA (Bowman et al. 1988). In *Arabidopsis*, ABA stimulates the accumulation of storage lipids (Finkelstein and Somerville 1990). Abscisic acid also participates in storage-protein synthesis during seed maturation. For instance, the *Arabidopsis abi3* mutant has only two-thirds of the wild-type level of storage proteins (Koornneef et al. 1989; Finkelstein and Somerville 1990). In maize, the synthesis of storage protein Gib1 was found to depend on ABA (Rivin and Grudt 1991). Interestingly, fragments of Gib1 protein accumulated late in embryogenesis, when ABA sensitivity is lost, which led the authors to suggest that ABA might also suppress storage-protein degradation.

There are many experimental conditions in which seeds or embryos seem to be carrying on both maturation- and germination-specific processes (Hughes and Galau 1991; Jakobsen et al. 1994). We would like to propose that both programs compete for the available resources, and that diverting resources towards reserve accumulation is a common principle underlying many of the effects of ABA in seed processes, including the prevention of germination.

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