Proteomics of Arabidopsis Seed Germination. A Comparative Study of Wild-Type and Gibberellin-Deficient Seeds[1]

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We examined the role of gibberellins (GAs) in germination of Arabidopsis seeds by a proteomic approach. For that purpose, we used two systems. The first system consisted of seeds of the GA-deficient *ga1* mutant, and the second corresponded to wild-type seeds incubated in paclobutrazol, a specific GA biosynthesis inhibitor. With both systems, radicle protrusion was strictly dependent on exogenous GAs. The proteomic analysis indicated that GAs do not participate in many processes involved in germination sensu stricto (prior to radicle protrusion), as, for example, the initial mobilization of seed protein and lipid reserves. Out of 46 protein changes detected during germination sensu stricto (1 d of incubation on water), only one, corresponding to the cytoskeleton component α-2,4 tubulin, appeared to depend on the action of GAs. An increase in this protein spot was noted for the wild-type seeds but not for the *ga1* seeds incubated for 1 d on water. In contrast, GAs appeared to be involved, directly or indirectly, in controlling the abundance of several proteins associated with radicle protrusion. This is the case for two isoforms of *S*-adenosyl-methionine (Ado-Met) synthetase, which catalyzes the formation of Ado-Met from Met and ATP. Owing to the housekeeping functions of Ado-Met, this event is presumably required for germination and seedling establishment, and might represent a major metabolic control of seedling establishment. GAs can also play a role in controlling the abundance of a β-glucosidase, which might be involved in the embryo cell wall loosening needed for cell elongation and radicle extension.

Maturation drying is the normal terminal event in the vast majority of seeds, after which they pass into a metabolically quiescent state where they may remain for many years and still retain their viability (Hoekstra et al., 2001). Upon hydration under suitable conditions, the seed, if not dormant, reactivates its metabolism and commences germination, giving rise to a new plant.

Seed germination can be divided into three phases, imbibition, increased metabolic activity, and initiation of growth, which loosely parallel the triphasic water uptake of dry mature seeds. Morphologically, initiation of growth corresponds to radicle emergence; subsequent growth is generally defined as seedling growth. By definition, germination sensu stricto incorporates those events that start with the uptake of water by the quiescent dry seed and terminate with the protrusion of the radicle and the elongation of the embryonic axis (Bewley, 1997). From physiological studies on a wide variety of species, including a number of mutants, it appears that gibberellins (GAs) play a key role in late stages of seed germination (Hilhorst and Karssen, 1988, 1992; Karssen et al., 1989; Hilhorst and Toorop, 1997; Yamaguchi et al., 1998; Richards et al., 2001). Thus, in plant species such as Arabidopsis and tomato (*Lycopersicon esculentum*), the strong alleles of GA-deficient mutants are unable to complete germination without exogenous GAs (Koornneef and van der Veen, 1980; Groot and Karssen, 1987). Furthermore, inhibitors of GA biosynthesis such as paclobutrazol (PAC) and tetcyclacis prevent radicle protrusion (Karssen et al., 1989; Nambara et al., 1991). Many studies have focused on the role of GAs in dormancy breakage (Metzger, 1983; Hilhorst and Karssen, 1988, 1992; Derkx and Karssen, 1993; Bianco et al., 1994; Yang et al., 1995; Toyomasu et al., 1998; Kamiya and Garcia-Martinez, 1999; Grappin et al., 2000) and in the mobilization of seed reserves during seedling establish-

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ment (Skadsen, 1998; Walker-Simmons, 2000; Gomez-Cadenas et al., 2001; Shen et al., 2001). In contrast, there are only few reports on the mode of action of GAs during the early events occurring during seed germination before radicle protrusion. Here, two main mechanisms have been documented. In the first one, the role of GAs would be to induce endosperm and seed coat weakening. This process is required for the germination of many species, as these tissues confer part of the mechanical resistance to radicle protrusion (Groot and Karssen, 1987; Groot et al., 1988; Leubner-Metzger et al., 1996; Bradford et al., 2000; Debeaujon and Koornneef, 2000). In the second mechanism, GAs would be involved in resumption of cell cycle activity during germination, as documented for example for tomato seeds (Liu et al., 1994).

We are interested in determining the biochemical and genetic mechanisms that regulate the transition from quiescence to highly active metabolism during germination and seedling establishment. To this end, we previously initiated a proteome analysis of the model plant Arabidopsis (Gallardo et al., 2001) for which a complete genome sequence is now available (The Arabidopsis Genome Initiative, 2000). The long-term objective of this work is to provide reference maps of seed proteins to focus on the effects of environmental changes and developmental stages during seed maturation, desiccation, and germination. Such an approach already proved successful in investigating differential protein expression in Arabidopsis upon environmental changes or mutations (Meurs et al., 1992; Santoni et al., 1994, 1997; Leymarie et al., 1996; for review, see Thiellement et al., 1999; Jacobs et al., 2000; van Wijk, 2001).

In the present study, we used this global approach to investigate the role of GAs in germination of Arabidopsis seeds. For this purpose, we characterized the proteome of GA-deficient Arabidopsis seeds in which GA deficiency is conferred by the *ga1* mutation (*ga1-1* allele). The *GA1* gene codes for the enzyme *ent*-copalyl diphosphate synthase that catalyzes the first step in the GA biosynthetic pathway (Sun and Kamiya, 1994). In addition, we analyzed the effect of PAC on the proteome of wild-type (WT) seeds during germination. Arabidopsis seed germination is highly sensitive to this compound (Debeaujon and Koornneef, 2000), which blocks GA biosynthesis and thereby radicle emergence through inhibition of the enzyme *ent*-kaurene oxydase (encoded by the gene *GA3*).

RESULTS

Preparation of Seed Samples

Under optimal conditions (25°C), radicle protrusion of the WT Arabidopsis seeds started at 1.4 d of imbibition and it took almost 1.8 d for 50% of the seeds to reach this phase (Table I). The addition of GA_{4+7} to the germination medium had very little effect on the germination of WT seeds (Table I). PAC totally repressed radicle protrusion. The addition of GA_{4+7} (Derkx et al., 1994) to the germination medium reversed this PAC inhibition. However, under these conditions, seed germination was somewhat more heterogeneous ($T_{50} \approx 2.2$ d) than in the absence of PAC (Table I). The *ga1* mutant seeds did not complete germination at all on water. In the presence of exogenous GA_{4+7}, the germination vigor of the *ga1* seeds was very close to that of the WT seeds ($T_{50} \approx$ 1.8 d; Table I).

Table I. *Effects of GAs and paclobutrazol on germination performance of Arabidopsis seeds*

T_1 represents the start of germination (time to reach 1% of germination ± SD), T_{50}, the time to reach 50% of germination (± SD), and G_{max}, the final percentage of germination (± SD). Germination assays were carried out with the WT seeds and *ga1* mutant seeds for up to 5 d in the presence of water, 100 μM GA_{4+7}, and/or 100 μM PAC.

Seeds/Conditions	T_1	T_{50}	G_{max}
	d		%
WT/water	1.44 ± 0.07	1.84 ± 0.01	100 ± 0
ga1/water	>5	>5	0
ga1/GA_{4+7}	1.52 ± 0.01	1.77 ± 0.02	100 ± 0
WT/GA_{4+7}	1.36 ± 0.09	1.74 ± 0.04	100 ± 0
WT/PAC	>5	>5	0
WT/PAC + GA_{4+7}	1.32 ± 0.07	2.20 ± 0.05	98.7 ± 1.3

Proteome Analyses

We characterized the proteome of the following Arabidopsis seed samples: WT and *ga1* mutant dry mature seeds; WT and *ga1* seeds incubated for up to 3 d in the absence or presence of GA_{4+7}; and WT seeds incubated for up to 3 d in PAC. A comparison of the proteome from WT and *ga1* dry mature seeds revealed six polypeptides that showed a substantially higher accumulation level in the *ga1* seeds than in the WT seeds (Fig. 1). They were all identified as 12S globulin precursors by MALDI-TOF analysis (Table II). Two of them (protein nos. 69 and 177 in Table II) exhibited experimental molecular masses substantially smaller than theoretically expected. It is possible that these polypeptides corresponded to proteolyzed fragments of the precursor globulin forms. There were no significant changes in the abundance of the other proteins present at the dry mature stage for both types of dry mature seeds (not shown).

A systematic comparison of two-dimensional gels for the various protein extracts allowed classifying seed proteins from their specific accumulation patterns. Some of them have been described previously (Gallardo et al., 2001); others were identified in the present study (Tables III and IV). Type-1 and -2 proteins corresponded to polypeptides whose abundance varied (up- and down-regulation, respectively) during germination (Figs. 2–4). Type-3 proteins showed a specific increase in their accumulation at the moment of radicle protrusion (Fig. 2C).

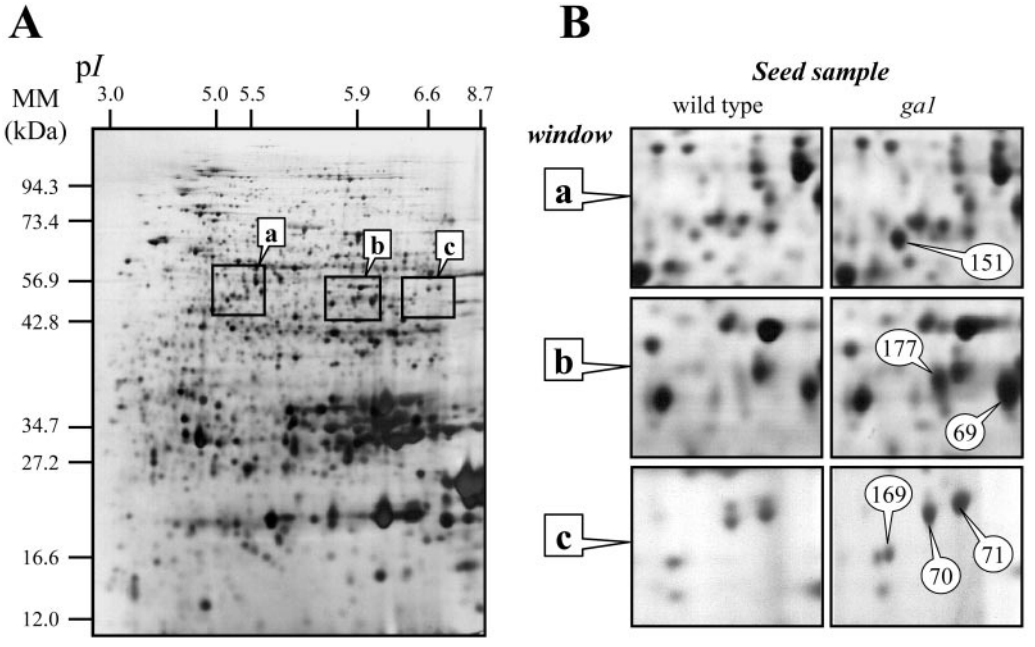

Figure 1. Characterization of Arabidopsis proteins whose abundance differed in dry mature seeds of WT and *ga1* mutant. An equal amount (200 μg) of total protein extracts was loaded in each gel. A, Silver-stained two-dimensional gel of total proteins from dry mature seeds of *ga1* mutant. The indicated portions of the gel, a through c, are reproduced in B. B, Enlarged windows, a through c, of two-dimensional gels as shown in A for WT seeds (left) and *ga1* mutant seeds (right). The six labeled protein spots (protein nos. 151, 177, 69, 169, 70, and 71) were identified by matrix-assisted laser desorption-ionization-time-of-flight (MALDI-TOF) analysis (see Table II). The figure shows representative experiments carried out at least five times. Protein spot quantitation was carried out as described in "Materials and Methods" from at least three gels for each seed sample.

Influence of GAs after 1 d of Imbibition

The proteome of *ga1* seeds was analyzed after 1 d of imbibition in water. This stage corresponded to germination sensu stricto for the WT seeds because none of the WT seeds showed radicle protrusion during this period (Table I). This analysis revealed in the imbibed *ga1* seeds 45 proteins whose abundance increased or decreased with respect to the dry mature state. All of them were also evidenced during 1 d of imbibition in water of the WT seeds. Moreover, their level of variation with respect to the dry mature state was generally very similar for the *ga1* and WT seeds (Table III; Figs. 3 and 4). The only significant difference between the two types of seeds was in a protein spot (no. 6 in Table III). The abundance of this protein strongly increased during 1 d of imbibition in water of the WT seeds, but not for the *ga1* seeds (Fig. 5). Incubation of the *ga1* seeds in the presence of GA_{4+7} for 1 d (none of these seeds completed germination under these conditions; Table I) entailed a large accumulation of this protein spot (Fig. 5). This protein was identified as α-2,4 tubulin by MALDI-TOF analysis (Table III).

The above results suggested that the abundance of α-2,4 tubulin is under GA control. To assess this possibility, the proteome of WT seeds was analyzed

Table II. *Arabidopsis polypeptides whose abundance was significantly higher in dry mature ga1 seeds than in dry mature WT seeds*

No.	Experimental Molecular Mass	Experimental pI	Arabidopsis Protein Name	Coverage	Theoretical Molecular Mass	Theoretical pI	Accession No.	Relative Abundance[a]
	kD			%	*kD*			
169	47.20	6.7	12S-1 Seed storage protein precursor	15	49.99	7.26	P15455	>200
151	45.59	5.39	Similar to 12S seed storage precursor	23	49.67	5.47	4204298	3.5 ± 0.1
177	42.94	6.12	12S-2 Seed storage protein precursor	14	50.64	6.77	112682	2.4 ± 0.1
71	50.44	7.67	12S-1 Seed storage protein precursor	22	49.99	7.26	P15455	2.4 ± 0.1
70	49.43	7.19	12S-1 Seed storage protein precursor	61	49.99	7.26	P15455	2.0 ± 0.1
69	43.67	6.29	12S-2 Seed storage protein precursor	18	48.03	6.56	P15456	1.8 ± 0.1

[a] Data obtained from densitometric analysis of individual spots (see Fig. 1): normalized spot volume in the mature dry *ga1* seeds divided by the normalized spot volume in the dry mature WT seeds ± SD (n = 3); >200 means that the accumulation level of the corresponding protein in the dry mature WT seeds was close to background.

Table III. *Arabidopsis polypeptides whose abundance specifically vary during 1 d of imbibition*

WT/water, WT seeds incubated for 1 d in water; WT/PAC, WT seeds incubated in 100 μM PAC; *ga1*/water, *ga1* mutant seeds incubated in water for 1 d as described in "Materials and Methods." Type-1 and -2 proteins, proteins whose accumulation level specifically increased or decreased during 1 d of imbibition, respectively. Postsource decay (PSD), protein identified by MALDI-TOF and PSD analyses.

Protein Type	No.	Experimental Molecular Mass	Experimental p*I*	Arabidopsis Protein Name	Coverage	Theoretical Molecular Mass	Theoretical p*I*	Accession No.	Relative Abundance[a]		
									WT/water	*ga1*/water	WT/PAC
		kD			%	*kD*					
1[b]	44	16.14	7.42	β-Subunit of a 12S seed storage protein	PSD	20.72	6.36	4204299	>200	>200	>200
1[c]	164	35.03	5.60	Similar to aspartic protease subunits	19	47.46[e]	7.97[e]	2160151	>200	>200	>200
1[c]	29	38.35	5.72	NADP-dependent P1 oxydoreductase	14	38.53	5.81	2498731	>200	>200	>200
1[d]	19	7.18	6.79						>200	>200	>200
1[b]	32	15.16	5.75	β-Subunit of 12S-2 seed storage protein (fragment)	18	20.80	7.03	P15456	24.0 ± 1.6	19.2 ± 2.9	10.5 ± 0.9
1[c]	28	36.72	5.60	ATP4A peroxidase	18	34.84	6.49	1429213	22.2 ± 0.9	32.1 ± 0.2	23.7 ± 1.0
1[b]	77	25.42	5.78	α-Subunit of 12S cruciferin (fragment)	30	34.68	6.42	T04623	7.9 ± 0.5	9.3 ± 0.7	8.7 ± 0.5
1[b]	76	23.94	5.58	α-Subunit of 12S cruciferin (fragment)	27	34.68	6.42	T04623	7.6 ± 1.4	8.4 ± 0.6	7.3 ± 0.2
1[c]	148	65.31	5.37	Mitochondrial heat shock protein 60	16	55.25	5.31	2924773	6.1 ± 1.5	3.4 ± 0.1	4.9 ± 0.8
1[b]	26	96.38	5.75	Cytoplasmic aconitate hydratase (aconitase)	19	98.15	5.79	4586021	6.0 ± 1.2	4.6 ± 0.4	6.3 ± 0.7
1[c]	33	14.86	5.87	α-Subunit of 12S-1 cruciferin (fragment)	18	29.11	6.49	P15455	5.9 ± 0.1	4.6 ± 0.2	3.8 ± 0.5
1[b]	6	54.71	5.06	α-2,4 Tubulin	58	49.54	4.93	320183	5.6 ± 0.4	1.0 ± 0.1	1.2 ± 0.2
1[d]	34	13.19	5.9						5.0 ± 0.1	3.3 ± 0.4	2.5 ± 0.2
1[b]	24	42.94	5.06	Actin 7	PSD	41.96	5.26	P53492	4.7 ± 0.1	6.2 ± 0.3	4.6 ± 0.2
1[c]	168	62.91	7.08	Glyoxysomal malate synthase	10	63.89	8.02	11268170	4.3 ± 0.4	3.4 ± 0.1	5.2 ± 0.5
1[b]	12	16.14	5.71	β-Subunit of 12S-2 seed storage protein (fragment)	30	20.80	7.03	P15456	4.1 ± 0.4	2.7 ± 0.4	3.7 ± 0.4
1[d]	30	31.24	5.39						4.1 ± 0.2	4.9 ± 0.6	5.9 ± 0.5
1[b]	89	15.80	5.72	β-Subunit of 12S-2 seed storage protein (fragment)	36	20.80	7.03	P15456	4.1 ± 0.9	3.0 ± 0.3	3.3 ± 0.8
1[c]	39	38.52	6.23	Cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	34	36.99	6.34	P25858	3.9 ± 0.1	5.1 ± 0.7	7.7 ± 0.1
1[d]	31	15.1	5.66						3.9 ± 0.1	2.3 ± 0.1	3.2 ± 0.1
1[d]	160	82.27	5.94	Cobalamin-independent Met synthase	12	84.36	6.09	8134566	3.9 ± 0.8	2.6 ± 0.2	3.7 ± 0.2
1[c]	13	72.97	6.30	Similar to mycoplasma-like organism proteins	15	67.23	9.55	4585879	3.6 ± 0.1	3.9 ± 0.3	3.5 ± 0.2
1[b]	17	73.40	6.58	Phospho*enol*pyruvate carboxykinase	17	73.40	6.61	7449802	3.5 ± 0.7	4.4 ± 0.7	5.6 ± 0.2
1[c]	159	23.54	5.33	α-Subunit of 12S cruciferin (fragment)	15	34.68	6.42	T04623	3.5 ± 0.7	3.5 ± 1.3	3.8 ± 0.1
1[c]	5	57.86	5.11	α-3,5 Tubulin	27	49.65	4.95	135406	3.3 ± 0.1	3.1 ± 0.2	3.4 ± 0.4
1[b]	41	37.72	6.74	WD-40 repeat protein	PSD	35.78	7.61	2289095	3.3 ± 0.2	4.4 ± 0.2	3.8 ± 0.7
1[d]	156	36.04	3.99						3.3 ± 0.1	2.5 ± 0.3	3.3 ± 0.1
1[b]	23	56.48	6.64	Catalase 2	34	56.91	6.63	P25819	3.0 ± 0.1	3.2 ± 0.1	3.2 ± 0.1
1[d]	163	40.75	5.55						2.7 ± 0.4	3.8 ± 0.3	3.1 ± 0.4
1[b]	4	57.35	4.89	β-2 tubulin	36	50.73	4.70	166898	2.4 ± 0.1	2.2 ± 0.2	2.2 ± 0.1
1[c]	149	64.96	5.18	Mitochondrial heat shock protein 60	15	57.65	5.19	12644189	2.4 ± 0.1	2.5 ± 0.2	2.1 ± 0.1
1[d]	176	39.27	6.33						2.1 ± 0.1	2.2 ± 0.1	1.9 ± 0.1
1[c]	40	38.55	6.26	Cytosolic GAPDH	26	36.99	6.34	P25858	1.7 ± 0.1	1.8 ± 0.2	1.7 ± 0.1
2[d]	7	37.55	5.09						<0.005	<0.005	<0.005
2[d]	21	59.83	6.57	Similar to 7S seed storage proteins	12	55.06	6.64	9279682	<0.005	<0.005	<0.005
2[c]	22	59.14	6.57	Similar to 7S seed storage proteins	12	55.06	6.64	9279682	<0.005	<0.005	<0.005
2[d]	35	56.47	6.84						<0.005	<0.005	<0.005
2[c]	36	40.42	6.44	Cytosolic GAPDH	34	36.99	6.34	P25858	<0.005	<0.005	<0.005
2[b]	37	40.29	6.49	Cytosolic GAPDH	26	36.99	6.34	P25858	<0.005	<0.005	<0.005
2[d]	152	57.98	4.99						<0.005	<0.005	<0.005
2[d]	154	26.29	4.86						<0.005	<0.005	<0.005
2[d]	155	25.23	4.86						<0.005	<0.005	<0.005
2[c]	161	52.11	5.71	Mitochondrial dihydrolipoamide *S*-acetyltransferase	10	45.07	5.93	9279589	<0.005	<0.005	<0.005
2[c]	170	38.56	7.13	WD-40 repeat protein	27	35.78	7.61	2289095	<0.005	<0.005	<0.005
2[c]	165	38.32	5.78	Acyl carrier protein enoyl reductase	36	33.16	5.84	4006834	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.01
2[d]	167	63.25	7.34						0.3 ± 0.1	0.5 ± 0.1	0.3 ± 0.1

[a] Data obtained from densitometric analysis of individual spots (see Fig. 2, A and B): normalized spot volume in the WT seeds incubated for 1 d in water (WT/water), in the *ga1* mutant seeds incubated for 1 d in water (*ga1*/water), or the WT seeds incubated for 1 d in 100 μM PAC (WT/PAC) divided by the normalized spot volume in the corresponding dry mature seeds ± SD ($n = 3$); >200 means that the accumulation level of the corresponding protein in the dry mature seeds was close to background; <0.005 means that the accumulation level of the corresponding protein in the 1-d imbibed seeds was close to background. [b] Polypeptides identified by Gallardo et al. (2001). [c] Polypeptides identified by MALDI-TOF and/or PSD analyses in the present study. [d] Polypeptides characterized in the present study, but not yet identified. [e] Size and p*I* values of the precursor form.

Table IV. *Arabidopsis proteins whose level increased during radicle protrusion (type-3 proteins) with the WT seeds incubated for 2 d in water*

PSD, Protein identified by MALDI-TOF and PSD analyses.

Protein Type	No.	Experimental Molecular Mass	Experimental p*I*	Arabidopsis Protein Name	Coverage	Theoretical Molecular Mass	Theoretical p*I*	Accession No.	Relative Abundance[a]
		kD			%	*kD*			
3[b]	63	57.09	6.11	Isocitrate lyase	30	50.42	6.29	113024	>200
3[b]	58	36.28	5.49	Jasmonate-inducible MBP	41	32.16	5.46	9279641	>200
3[c]	62	43.25	5.67	Ado-Met synthetase 2	30	43.25	5.67	127045	>200
3[b]	67	63.25	6.47	Thioglucosidase (myrosinase)	PSD	60.26	6.91	S57621	>200
3[c]	47	66.57	5.33	Cytosolic phosphoglyceromutase	17	60.67	5.32	11133828	>200
3[b]	49	42.67	5.51	Ado-Met synthetase	19	42.79	5.51	9229983	18 ± 1
3[d]	65	29.65	5.82	α-Subunit of CRA1 cruciferin	24	29.23	6.49	81604	8.5 ± 0.1
3[b]	50	42.94	5.43	Chloroplastic translation elongation factor EF-Tu (mature form)	23	44.72	5.32	S09152	5 ± 1
3[c]	179	37.67	5.88	Mitochondrial malate dehydrogenase	PSD	33.9	6.00	11133715	4.0 ± 0.3
3[c]	73	42.28	6.47	β-Glucosidase	18	60.01	6.20	9294684	4.0 ± 0.4
3[b]	66	62.82	6.33	Jasmonate-inducible protein	16	62.00	6.00	8777418	3.1 ± 0.2

[a] Data obtained from densitometric analysis of individual spots (see Figs. 2C and 6): normalized spot volume in the 2-d imbibed seeds divided by the normalized spot volume in the 1-d imbibed seeds ± SD ($n = 3$); >200 means that the accumulation level of the corresponding protein in the 1-d imbibed seeds was close to background. [b] Polypeptides identified by Gallardo et al. (2001). [c] Polypeptides identified by MALDI-TOF and/or PSD analyses in the present study.

after 1 d of imbibition in 100 μM PAC. In these conditions, the abundance of α-2,4 tubulin did not increase, but remained at a constant low level similar to the *ga1* seeds incubated on water (Fig. 5).

Aside from the α-2,4 tubulin spot, this analysis also revealed in the PAC-treated seeds the whole set of 45 protein spots that differentially accumulated, as during 1 d of imbibition in water of the WT and *ga1* seeds (Table III; Figs. 3 and 4).

Influence of GAs after 2 d of Imbibition

Following 2 d of imbibition in water, about 80% of the WT seeds completed germination (Table I).

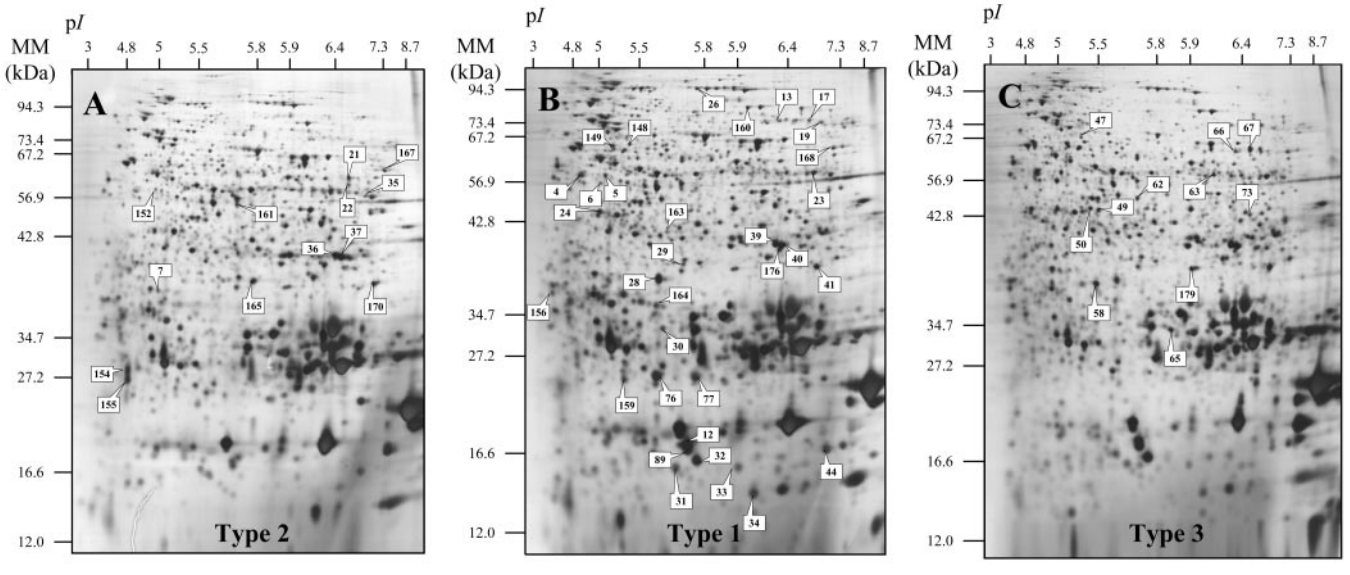

Figure 2. Reference maps for WT Arabidopsis seed proteins whose abundance specifically vary during germination (1 d of imbibition in water) and radicle protrusion (2 d of imbibition in water). An equal amount (200 μg) of total protein extracts was loaded in each gel. The figure shows representative experiments carried out at least five times. A, Silver-stained two-dimensional gel of total proteins from dry mature seeds showing the type-2 proteins (labeled) whose abundance specifically decreased during germination. The labeled proteins are listed in Table III. B, Silver-stained two-dimensional gel of total proteins from 1-d imbibed seeds showing the type-1 proteins (labeled) whose abundance specifically increased during germination. The labeled proteins are listed in Table III. C, Silver-stained two-dimensional gel of total proteins from 2-d imbibed seeds showing the type-3 proteins (labeled) whose abundance specifically increased during radicle protrusion. The labeled proteins are listed in Table IV.

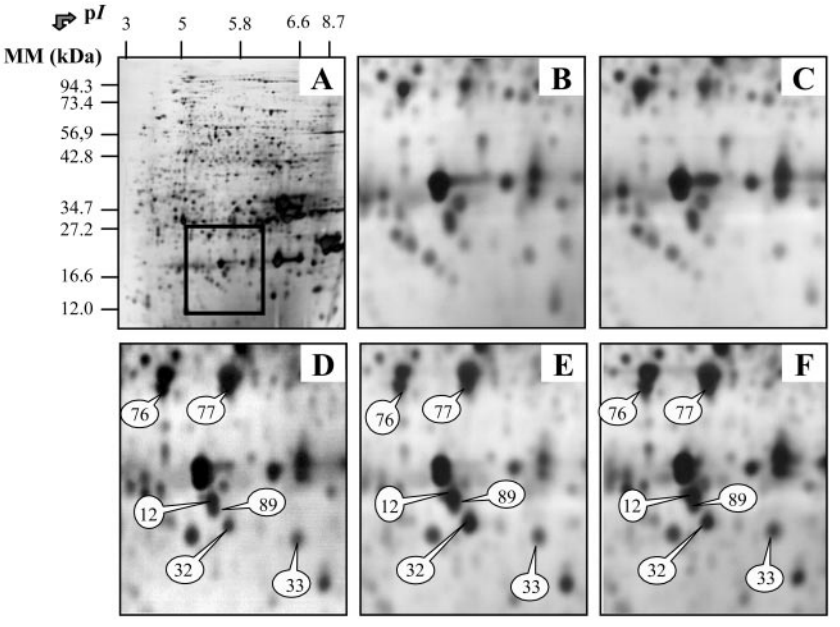

Figure 3. Mobilization of seed storage proteins during 1 d of imbibition. An equal amount (200 μg) of total protein extracts was loaded in each gel. The figure shows representative experiments carried out at least five times. A, Silver-stained two-dimensional gel of total proteins from dry mature WT seeds. The indicated portion of the gel is reproduced in B through F for the following seed samples. B, Dry mature WT seeds. C, Dry mature *ga1* seeds. D, WT seeds incubated for 1 d in 100 μM PAC (none of the seeds germinated under these conditions; see Table I). E, WT seeds incubated for 1 d in water (none of the seeds germinated under these conditions; see Table I). F, *ga1* mutant seeds incubated for 1 d in water (none of the seeds germinated under these conditions; see Table I). The labeled type-1 proteins (whose abundance increased during 1 d of imbibition for the three seed samples) are listed in Table III. They correspond to fragments of 12S cruciferin α-subunits (protein nos. 33, 76, and 77) and β-subunits (protein nos. 12, 32, and 89).

Therefore, this stage corresponded to radicle protrusion. Proteome analysis revealed a set of 11 type-3 proteins whose abundance increased with respect to dry mature seeds (Fig. 2C; Table IV). None of them could be evidenced upon incubation of the *ga1* seeds for 2 d in water, during which none of the mutant seeds showed radicle protrusion (Fig. 6). An absence of further modification of the proteome was also observed upon incubation of the WT seeds for 2 d in PAC (Fig. 6), which prevented radicle protrusion (Table I).

Influence of GAs after 3 d of Seed Imbibition

The proteome of *ga1* seeds was analyzed after 3 d of imbibition in water, which period corresponded to seedling establishment for the WT seeds incubated on water (Table I). In the still non-germinating *ga1* seeds, there were no further protein changes compared with the situation observed with *ga1* seeds imbibed for 1 and 2 d in water (Fig. 7). This constancy in the proteome imposed by a GA deficiency was also confirmed by analyzing the protein extracts from the WT seeds incubated for 3 d in PAC solution (Fig. 7).

DISCUSSION

To characterize the proteins and mechanisms that are under GA control during germination, our present objective was to use the reference map of

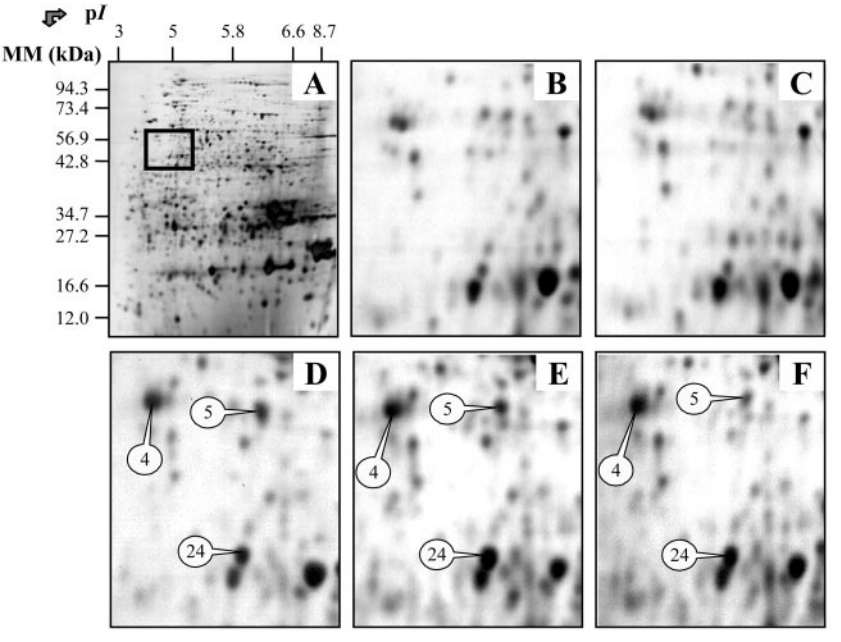

Figure 4. Characterization of some cytoskeleton protein components whose abundance increased during 1 d of imbibition (type-1 proteins). An equal amount (200 μg) of total protein extracts was loaded in each gel. The figure shows representative experiments carried out at least five times. A, Silver-stained two-dimensional gel of total proteins from dry mature WT seeds. The indicated portion of the gel is reproduced in B through F for the following seed samples. B, Dry mature WT seeds. C, Dry mature *ga1* seeds. D, WT seeds incubated for 1 d in 100 μM PAC (none of the seeds germinated under these conditions; see Table I). E, WT seeds incubated for 1 d in water (none of the seeds germinated under these conditions; see Table I). F, *ga1* mutant seeds incubated for 1 d in water (none of the seeds germinated under these conditions; see Table I). The labeled proteins are listed in Table III. They correspond to type-1 protein numbers 4 (β-tubulin), 5 (α-3, 5 tubulin), and 24 (actin 7).

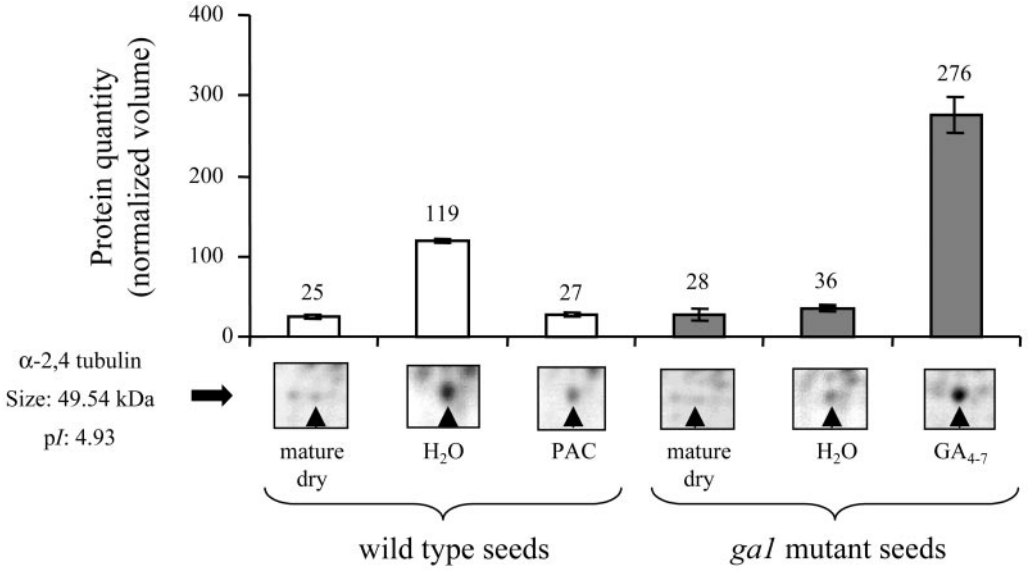

Figure 5. Quantitation of the accumulation level of α-2,4 tubulin (type-1 protein no. 6 in Table III) during 1 d of imbibition. The theoretical molecular mass and pI of α-2,4-tubulin are 49.54 kD and 4.93, respectively (Table III). The results are expressed as normalized volumes of the α-2,4 tubulin spot (± SD; $n = 3$) after 1 d of imbibition in water or in the presence of 100 μM PAC or 100 μM GA_{4+7}. A portion of an area of silver-stained two-dimensional gels is shown under the graph. The arrows point to the position of the α-2,4 tubulin spot.

Arabidopsis seed proteins initiated in previous work (Gallardo et al., 2001) and developed further in the present study. Toward this goal, we used two systems. The first consisted of the *ga1* mutant seeds (Koornneef and van der Veen, 1980). The second corresponded to the WT seeds incubated in PAC, a specific GA biosynthesis inhibitor. With both systems, radicle protrusion was strictly dependent on exogenous GAs, as previously reported (Koornneef and van der Veen, 1980; Karssen and Laçka, 1986; Debeaujon and Koornneef, 2000).

The present study indicates that whatever the system used to induce a GA deficiency, GAs seemed to control only few events associated with germination. Thus, the proteome of the *ga1* seeds incubated for 1 d in water revealed most of the protein changes evidenced for the WT seeds incubated under same experimental conditions. Similar behavior was observed during incubation of the WT seeds in the presence of PAC. In contrast, the realization of the events resulting in radicle protrusion appeared to depend on GA action. In both cases of GA deficiency, the proteome was blocked in a germination mode and never evolved toward a proteome characteristic of radicle protrusion, even for prolonged incubations (Figs. 6 and 7). The results will be discussed in the following sections.

Accumulation of Stored Protein Reserves in Mature Seeds and Their Mobilization during Germination

11–12S globulins are abundant seed storage proteins, which are widely distributed among higher plants. They are synthesized during seed maturation on the mother plant in a precursor form consisting of a single protein chain of about 60 kD. At later stages, the precursor form is cleaved, yielding the mature globulins generally found in storage protein bodies of dry mature seeds. These are composed of six subunit pairs that interact noncovalently. Each of these pairs consists of an acidic α-subunit of $M_r \approx 40{,}000$ and a basic β-subunit of $M_r \approx 20{,}000$ that are covalently joined by a single disulfide group. These subunits are subsequently broken down during germination and used by the germinating seedling as an initial food source (Bewley and Black, 1994; Shewry et al., 1995).

The present study disclosed six proteins that were differentially accumulated in dry mature *ga1* seeds (Table II; Fig. 1). They corresponded to 12S cruciferin precursors, meaning that they were not cleaved during seed development to yield the mature α- and β-globulin subunits. The synthesis of seed storage proteins is controlled by the phytohormone abscisic acid (ABA) during seed maturation (Finkelstein et al., 1985; Bustos et al., 1998). However, GAs could also be involved in this regulation, as suggested by the observation that they are also present in developing maize (*Zea mays*) embryos (White and Rivin, 2000; White et al., 2000). Here, not simply ABA, but rather mutual antagonism of GAs and ABA, appears to govern the choice between precocious germination or quiescence and maturation. Thus, a GA deficiency early in maize embryo development, induced genetically or via biosynthesis inhibitors, mimicked the effects of exogenous ABA, as inferred by the suppression of precocious germination, the acquisition of anthocyanin pigments, and the accumulation of a

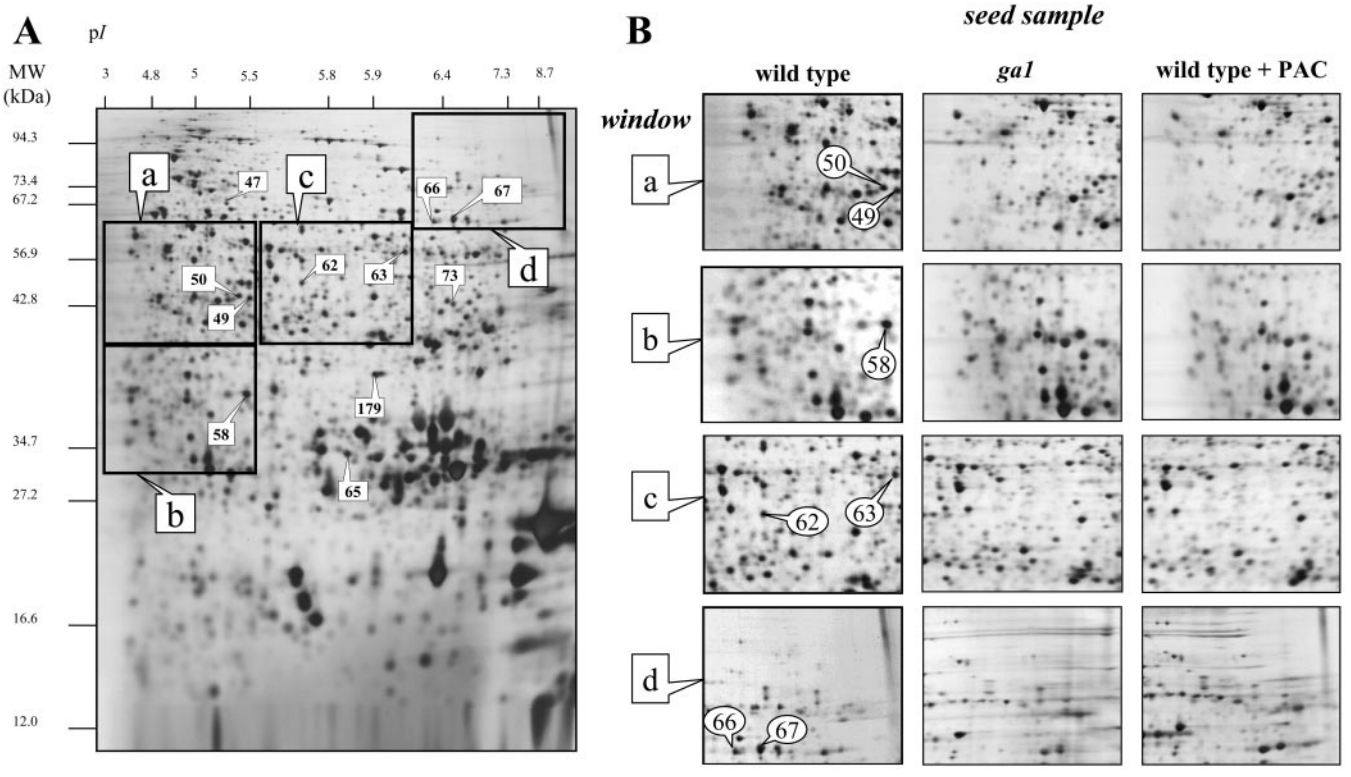

Figure 6. Evolution of the seed proteome in WT and *ga1* mutant seeds incubated for up to 2 d in water and in WT seeds incubated for up to 2d in PAC, which period corresponded to radicle protrusion for the WT seeds incubated on water (see Table I). An equal amount (200 μg) of total protein extracts was loaded in each gel. The figure shows representative experiments carried out at least five times. A, Silver-stained two-dimensional gel of total proteins from WT seeds incubated for 2 d in water showing the type-3 proteins (labeled) whose abundance specifically increased during radicle protrusion. The labeled proteins are listed in Table IV. The indicated portions of the gel, a through d, are reproduced in B. B, enlarged windows, a through d, of two-dimensional gels as shown in A for WT seeds incubated for 2 d in water (left), *ga1* mutant seeds incubated for 2 d in water (middle; none of the seeds germinated under these conditions; see Table I), and WT seeds incubated for 2 d in 100 μM PAC (right; none of the seeds germinated under these conditions; see Table I). The labeled proteins are listed in Table IV.

variety of maturation-phase mRNAs (White and Rivin, 2000). Therefore, a GA deficiency, as occurs in *ga1* Arabidopsis mutant plants, could explain the sustained synthesis of 12S cruciferin precursors, up to a stage at which they are not normally expected to accumulate. There exists a TAACAAA sequence in position −276 of the *CRA1* gene encoding 12S-2 globulin (spot nos. 69 and 177 in Table II). This sequence is similar to the GA response element (GARE) TAACAA/GA motif, which is known to be involved in GA-responsive gene expression (Lovegrove and Hooley, 2000). Thus, our data support the view that GAs play a role in the accumulation of seed storage proteins during maturation, presumably by specifically impeding the accumulation of 12S globulin precursors. The processing of these precursor forms affects the accumulation of 12S globulins within protein bodies because unprocessed forms can only associate as trimers, whereas mature globulins associate as hexamers (Shewry and Casey, 1999). However, we note that the *ga1* mutant plants experienced the action of exogenous GAs during their growth and development; these plants were sprayed once a week with 10 μM GA_{4+7} to stimulate elongation growth and anther development. This suggests that the action of exogenous GAs did not completely mimic that of endogenous GAs in terms of concentrations and/or spatial and temporal patterns of expression. The presence of mature 12S storage proteins in the *ga1* seeds next to relatively higher levels of the precursor forms suggest that residual exogenous GAs were sufficient to induce cleavage of the precursor molecules or that the cleavage is only partly under GA control. The relatively higher levels of precursors indicate at least some role for GAs in the final phase of storage protein synthesis.

Mobilization of the mature forms of 12S globulins already begins during germination and continues during seedling establishment (Job et al., 1997; Gallardo et al., 2001). As the same accumulation of proteolyzed forms (fragments) of cruciferin subunits occurred during 1 d of imbibition in water of the WT and *ga1* mutant seeds (Fig. 3; Table III), the present study shows that the initial mobilization of globulin subunits during germination was not dependent upon GA action. This implies that synthesis and/or

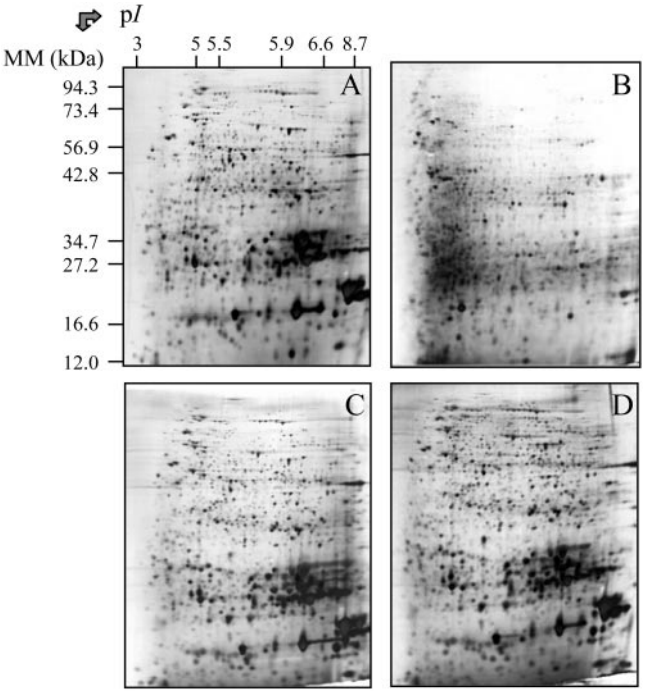

Figure 7. Evolution of the seed proteome in WT and *ga1* mutant seeds incubated for up to 3 d in water and in WT seeds incubated for up to 3 d in PAC. An equal amount (200 μg) of total protein extracts was loaded in each gel. The figure shows representative experiments carried out at least five times. A, Silver-stained two-dimensional gel of total proteins from dry mature WT seeds. B, Silver-stained two-dimensional gel of total proteins from WT seeds incubated in water for 3 d corresponding to seedling establishment. C, Silver-stained two-dimensional gel of total proteins from *ga1* mutant seeds incubated for 3 d in water. D, Silver-stained two-dimensional gel of total proteins from WT seeds incubated in 100 μM PAC for 3 d. The figure shows that the proteome of the *ga1* mutant seeds incubated for 3 d in water (C) and that of the WT seeds incubated for 3 d in PAC (D) did not exhibit the characteristic changes observed with the WT seeds incubated in water (B). Note in particular the massive loss of low-M_r proteins in the 5.9 to 8.7 pI range observed during the 3 d of imbibition in water of the WT seeds (compare A and B), but not with the *ga1* mutant seeds (C) and the WT seeds incubated in PAC (D).

activation of the proteases that are involved in the initial mobilization of 12S cruciferin subunits during germination are not controlled by GAs. In agreement, a polypeptide corresponding to an aspartic protease subunit accumulated during 1 d of imbibition of the *ga1* mutant seeds as well (type-1 protein no. 164 in Table III). However, whereas cruciferin mobilization continued in the WT seeds during the radicle protrusion step and seedling establishment (Table IV; Gallardo et al., 2001), this mobilization did not continue beyond germination for the *ga1* seeds incubated in water. This suggests that further cruciferin mobilization depended on GA action.

Mobilization of Stored Lipid Seed Reserves

In oilseeds, a massive conversion of triacylglycerols (the major storage lipids in these seeds; Miquel and Browse, 1995) to sugar occurs after germination (Beevers, 1980). Within the entire gluconeogenic pathway, the conversion of fatty acid to succinate takes place within the glyoxysomes, which contain enzymes for fatty acid β-oxidation and the glyoxylate cycle.

Consistent with previous results (Comai et al., 1989; Turley and Trelease, 1990; Gallardo et al., 2001), several enzymes in this pathway, such as aconitase, malate synthase, catalase, and phospho*enol*pyruvate carboxykinase, accumulated prior to radicle protrusion during germination of the WT seeds (protein nos. 26, 168, 23, and 17 in Table III, respectively). The same pattern of behavior was observed during 1 d of imbibition in water of the *ga1* seeds (Table III). In addition, PAC did not prevent the accumulation of these four enzymes in the WT seeds (Table III). Thus, in none of these four enzymes is the accumulation level under GA control. For the WT seeds, other enzymes in this pathway accumulate later, at the moment of radicle protrusion, as occurs for isocitrate lyase, phosphoglyceromutase, and the mitochondrial enzyme malate dehydrogenase (protein nos. 63, 47, and 179 in Table IV, respectively). This accumulation pattern was not seen for the *ga1* seeds incubated for up to 3 d in water, and for the WT incubated for up to 3 d in PAC. This suggests that accumulation of these three enzymes was dependent on GA action. Marriott and Northcote (1975) showed that GAs stimulate the induction of isocitrate lyase activity during germination. Moreover, a TAACAAA sequence analogous to the GARE is present in position −1403 and −233 of the genes encoding isocitrate lyase and phosphoglyceromutase, respectively. These findings are consistent with the hypothesis that these two genes may be directly controlled by GAs. In contrast, the 5′-upstream region of the *NAD-MDH* gene encoding the mitochondrial malate dehydrogenase does not contain a potential GARE, suggesting that GAs indirectly control expression of this gene. A recent characterization of two allelic Arabidopsis mutants, *icl-1* and *icl-2*, which lack the glyoxylate cycle because of the absence of the key enzyme isocitrate lyase, demonstrated that the glyoxylate cycle is not essential for germination, but is important for seedling establishment and survival (Eastmond et al., 2000).

Cell Cycle Activity

Considerable experimental evidence compels the view that resumption of cell cycle activity is a specific feature of early germination (Georgieva et al., 1994; Górnik et al., 1997; Özbingöl et al., 1999; de Castro et al., 2000; Vázquez-Ramos, 2000). Our previous work (Gallardo et al., 2001) and the present study revealed an accumulation of five proteins associated with cell cycle events during germination of the WT Arabidopsis seeds. These proteins were identified to as actin 7, α-2,4 tubulin, α-3,5 tubulin, β-tubulin, and a

WD-40 repeat protein (protein nos. 24, 6, 5, 4, and 41 in Table III, respectively; Figs. 4 and 5). Tubulins are associated with cell division and cell enlargement aspects of the cell cycle. During cell division, they play an important role in separation of the organelles and daughter chromosomes (mitosis). An accumulation of β-tubulin during early germination has repeatedly been observed in many species (see de Castro et al., 2000). With germinating tomato and cucumber (*Cucumis sativus*) seeds, cortical microtubules are formed in the radicle prior to protrusion. These cortical microtubules are most likely associated with preparation of cell elongation.

Liu et al. (1994) demonstrated a GA requirement for resumption of cell cycle activity during germination of tomato seeds. From the present results, out of the five above-mentioned proteins, only α-2,4 tubulin showed a distinct pattern of accumulation when comparing the *ga1* mutant and the WT Arabidopsis seeds after 1 d of imbibition in water. This protein strongly accumulated in the WT seeds, but not in the *ga1* mutant seeds (Fig. 5). In addition, in the WT seeds PAC mimicked this specific defect in the accumulation of α-2,4 tubulin (Fig. 5). Moreover, an accumulation of this protein occurred during 1 d of imbibition of the *ga1* seeds in GA_{4+7} solution (Fig. 5), whereas none of the seeds had completed germination under these conditions (Table I). Therefore, all these data support the conclusion that GAs control the accumulation of α-2,4 tubulin during germination. It is interesting that the accumulation of α-3,5 tubulin or that of β-tubulin was not influenced by GA deficiency induced genetically or by the PAC treatment (Table III; Fig. 4). The same behavior was also observed for another component of the cytoskeleton, actin 7 (Table III; Fig. 4), which is the sole form of actin in germinating seeds (McDowell et al., 1996). Our data indicate that in Arabidopsis, only the accumulation of part of the cytoskeleton components is under GA control.

In Arabidopsis, two genes, *TUA2* and *TUA4*, encode α-2,4 tubulin (Kopczak et al., 1992). They both encode for exactly the same protein sequence. We found no evidence for the existence of regulatory motifs equivalent to the GARE in the 5′-upstream region of these two genes. This might suggest that the expression of the *TUA2* and *TUA4* genes are not directly controlled by GAs during germination. It has been shown that α-tubulin can acquire post-translational modifications such as acetylation in the tobacco (*Nicotiana tabacum*) pollen tube microtubules, presumably to stabilize the microtubular network (Astrom, 1992). Furthermore, Huang and Lloyd (1999) showed that GAs stimulate α-tubulin acetylation and stabilize microtubules in maize suspension cells. It will be interesting to reinvestigate the mechanism of α-2,4 tubulin accumulation during germination in the context of this specific post-translational process. In an alternate manner, GAs might exert their inducing effect in an indirect manner, for instance through loosening of the mechanical restraint. GA-deficient Arabidopsis *ga-1* seeds can germinate in water after removal of the surrounding testa and endosperm layers, or in a genetic background of *tt* (with transparent testa) or *rga* (for repressor of *gal-3*) and grow up to dwarf plants (Silverstone et al., 1997; Debeaujon and Koornneef, 2000). Cell cycle activity is a prerequisite for growth and organ formation. It is apparent that GAs are not essential for cell cycle activity.

Proteins Associated with Radicle Emergence

In agreement with previous work (Gallardo et al., 2001), the proteomic approach identified the existence of 11 type-3 proteins that are associated with the radicle protrusion step (Figs. 2C and 6; Table IV). These include the mature form of the plastidial translation elongation factor EF-Tu, two isoforms of *S*-adenosyl-Met (Ado-Met) synthetase, a β-glucosidase, and three proteins involved in defense mechanisms against pathogens. None of them could be detected in the proteome of *ga1* seeds incubated for up to 3 d in water or in that of the WT seeds incubated for up to 3 d in PAC solution. Therefore, the genes encoding these proteins are likely candidates for being regulated by GAs during radicle protrusion.

The 5′-upstream region of the nuclear gene encoding the plastidial translation elongation factor EF-Tu (protein no. 50 in Figs. 2C and 6) does not contain potential GARE. This might suggest that the expression of this gene is not directly controlled by GAs during radicle protrusion. Plastid differentiation is an early event in seed germination (Harrak et al., 1995). One possibility could be that plastid differentiation could not proceed when radicle protrusion is blocked because of GA deficiency. In accordance with this, an accumulation of EF-Tu during radicle protrusion would be developmentally regulated rather than being directly controlled by GAs.

Two of the type-3 proteins, protein numbers 49 and 62 in Table IV, corresponded to isoforms of Ado-Met synthetase. This enzyme catalyzes the formation of Ado-Met from Met and ATP. Ado-Met is the methyl donor in a myriad of transmethylation reactions. It is also involved in several reactions that are essential for plant growth and development, such as the biosynthesis of ethylene, spermidine, spermine, and biotin (Ravanel et al., 1998a; Hanson and Roje, 2001). Owing to these housekeeping functions, Ado-Met synthetase is presumably required for germination. In agreement, DL-propargyl-Gly, which is a potent and selective inhibitor of Met synthesis in plants (Ravanel et al., 1998b), substantially retarded radicle protrusion during Arabidopsis seed germination and totally repressed seedling growth (Gallardo, 2001). Mathur et al. (1992) showed that during germination GAs regulate the biosynthesis of two isoforms of

Ado-Met synthetase in the aleurone layer of wheat (*Triticum aestivum*) embryos. Furthermore, potential GARE are present in the 5′-upstream region of the genes encoding Ado-Met synthetase in Arabidopsis. A TAACAAA sequence is located in position −83 of the gene (identification no. MGD8.23) encoding the Ado-Met synthetase isoform corresponding to protein number 49 in Figures 2C and 6 and Table IV. This element is also present in position −99 of the *SAM2* gene encoding the second isoform (protein no. 62 in Figs. 2C and 6; Table IV). These features suggest that GAs can exert a direct control on the accumulation level of Ado-Met synthetase during the radicle protrusion step in Arabidopsis. Owing to the housekeeping functions of Ado-Met synthetase, this might represent a major control of seedling establishment. In the absence of this enzyme, the cell metabolism would be blocked.

A type-3 protein corresponded to a β-glucosidase (protein no. 73 in Table IV). This enzyme might be part of the group of various proteins that are involved in hydrolysis of the endosperm surrounding the root tip. In tomato and tobacco, this tissue confers part of the mechanical resistance to radicle protrusion (Groot and Karssen, 1987; Groot et al., 1988; Leubner-Metzger et al., 1996; Bradford et al., 2000). Also in Arabidopsis, weakening is likely under GA control (Debeaujon and Koornneef, 2000). In agreement, a potential GARE of sequence TAACAGA is located at position −288 of the Arabidopsis gene encoding this β-glucosidase (identification no. MHC9.5). The β-glucosidase protein might also, or alternatively, be involved in the embryo cell wall loosening needed for cell elongation and radicle extension.

Three type-3 proteins corresponded to a myrosinase (protein no. 67 in Figs. 2C and 6; Table IV) and two proteins, myrosinase-binding proteins (MBPs; protein nos. 58 and 66 in Figs. 2C and 6; Table IV). Myrosinase catalyzes the hydrolysis of glucosinolates, a group of sulfur-containing glycosides (Bones, 1990). Their breakdown products have important biological effects, as for the goitrogenic species that perturb thyroid function or the very reactive isothiocyanates that present antibacterial and antifungal properties (Rask et al., 2000). Myrosinases can associate with MBPs, presumably to regulate their activity (Rask et al., 2000). A potential GARE of TAACAAA sequence is present in position −1087 and −831 of the genes coding the two MBPS corresponding to protein numbers 58 and 66 (Figs. 2C and 6; Table IV), respectively. In contrast, no potential GARE could be found in the 5′-upstream region of the gene encoding the myrosinase protein (identification no. F3L24.13). Thus, these specific defense mechanisms that are important for seedling survival following germination (Rask et al., 2000) would be subjected to complex regulatory influences, possibly involving GA action (MBPs) and developmental regulation (myrosinase).

CONCLUSIONS

The present proteomic approach allowed us to identify several proteins whose abundance somehow depends on the action of GAs during the various phases of seed germination and seedling establishment in Arabidopsis. For example, this analysis demonstrated a specific involvement of GAs in regulation of the abundance of a cytoskeleton component, α-2,4 tubulin. However, GAs do not seem to participate directly in a number of processes involved in germination sensu stricto, as, for example, in the initial mobilization of seed protein and lipid reserves. Rather, it appears that GAs might be involved in controlling the accumulation levels of proteins associated with radicle protrusion and postgermination processes. The finding that potential GARE are located in the 5′-upstream region of several genes induced at the moment of radicle protrusion is in agreement with this proposal.

The present work further illustrates that proteomics can provide global information over a multitude of processes occurring during seed germination. These data can be analyzed further in combination with cDNA microarray technology (Girke et al., 2000; Wang et al., 2000; Schaffer et al., 2001; Seki et al., 2001), which will indicate whether gene regulation is controlled at the level of transcription or translation and protein accumulation. White et al. (2000) recently described a new set of expressed sequence tags from developing Arabidopsis seeds. Because these data and the microarray data of Girke et al. (2000) dealt with seed maturation, they cannot be directly compared with our present results on seed germination in terms of profiling of gene expression. However, it is worth noting that in addition to highly expressed storage protein genes, genomic and proteomic approaches can reveal a number of genes whose expression varies during seed development programs as for genes involved in cell cycle activity (e.g. actin 7 and tubulin chains) and in metabolism (e.g. Ado-Met synthetase). Protein function can be further studied by a combination of forward and reverse genetics (Aarts et al., 1995; Dubreucq et al., 1996; Pereira and Aarts, 1998) and proteomics, as has already been demonstrated in yeast (*Saccharomyces cerevisiae*) and *Escherichia coli*. These global expression-profiling approaches may prove useful for providing new information regarding genes involved in seed quality (Groot et al., 2000) and for characterizing novel chemicals acting positively (growth stimulants) or negatively (herbicides) on seedling establishment.

MATERIALS AND METHODS

Germination Experiments

Non-dormant seeds of Arabidopsis, ecotype Landsberg *erecta*, are referred to as WT seeds in this work. They were from the same seed lot as that used in a previous study

(Gallardo et al., 2001). The isolation of the non-germinating GA-deficient mutants *ga1-1* (W58) in the Landsberg *erecta* background was described by Koornneef and van der Veen (1980), and the molecular defects of these alleles was described by Sun et al. (1992). Germination assays were carried out on three replicates of 100 seeds. Seeds were incubated at 25°C, with 8 h of light daily, on three sheets of absorbent paper (Roundfilter paper circles, Ø 45 mm; Schleicher & Schuell, Dassel, Germany) and a black membrane filter with a white grid (ME 25/31, Ø 45 mm; Schleicher & Schuell) wetted with 1.3 mL of distilled water, in covered plastic boxes (Ø 50 mm). Assays were carried out in the presence or absence of 100 μM PAC (Greyhound Chromatography and Allied Chemicals, Birkenhead, Merseyside, UK) and/or 100 μM GA_{4+7} (Plant Protection, Fernhurst, UK). A seed was regarded as germinated when the radicle protruded through the seed coat.

Preparation of Total Protein Extracts

Total protein extracts were prepared from dry mature seeds and from seeds at different stages of germination. Following the grinding of seeds using mortar and pestle (with 150 mg representing approximately 8,400 WT seeds) in liquid nitrogen, total proteins were extracted at 2°C in 1.2 mL of thiourea/urea lysis buffer (Harder et al., 1999) containing 7 M urea (Amersham Pharmacia Biotech, Orsay, France), 2 M thiourea (Merck, Lyon, France), 4% (w/v) CHAPS (Amersham Pharmacia Biotech), and 1% (v/v) Pharmalyte, pH 3 to 10, carrier ampholytes (Amersham Pharmacia Biotech). This extraction buffer also contained 18 mM Tris-HCl (Trizma HCl; Sigma, St. Quentin Fallavier, France), 14 mM Trizma base (Sigma), the protease inhibitor cocktail "complete Mini" from Roche Diagnostics (Mannheim, Germany), 53 units mL^{-1} DNase I (Roche Diagnostics), 4.9 Kunitz units mL^{-1} RNase A (Sigma), and 0.2% (v/v) Triton X-100. After 10 min at 4°C, 14 mM dithiothreitol (DTT; Amersham Pharmacia Biotech) was added and the protein extracts were stirred for 20 min at 4°C and were then centrifuged (35,000*g* for 10 min) at 4°C. The supernatant was submitted to a second clarifying centrifugation as above. The final supernatant corresponded to the total protein extract. Protein concentrations in the various extracts were measured according to Bradford (1976). Bovine serum albumin was used as a standard.

Two-Dimensional Electrophoresis

Proteins were first separated by electrophoresis according to charge. Isoelectrofocusing was carried out with protein samples with an equivalent to an extract of 133 seeds corresponding to about 200 μg of protein for all samples. Proteins from the various extracts were separated using gel strips forming an immobilized nonlinear pH gradient from 3 to 10 (Immobiline DryStrip, pH 3–10 NL, 18 cm; Amersham Pharmacia Biotech). Strips were rehydrated for 14 h at 22°C with the thiourea/urea lysis buffer containing 2% (v/v) Triton X-100, 20 mM DTT, and the protein extracts. Isoelectrofocusing was performed at 22°C in the Multiphor II system (Amersham Pharmacia Biotech) for 1 h at 300 V and for 7 h at 3,500 V. Proteins were then separated according to size. Prior to the second dimension, the gel strips were equilibrated for 2 × 20 min in 2 × 100 mL of equilibration solution containing 6 M urea, 30% (v/v) glycerol, 2.5% (w/v) SDS, 0.15 M bis-Tris, and 0.1 M HCl (Görg et al., 1987; Harder et al., 1999). DTT (50 mM) was added to the first equilibration solution, and iodoacetamide (4% [w/v]) was added to the second (Harder et al., 1999). Equilibrated gel strips were placed on top of vertical polyacrylamide gels (10% [v/v] acrylamide, 0.33% [w/v] piperazine diacrylamide, 0.18 M Trizma base, 0.166 M HCl, 0.07% [w/v] ammonium persulfate, and 0.035% [v/v] Temed). A denaturing solution (1% [w/v] low-melting agarose [Invitrogen, Cergy-Pontoise, France], 0.4% [w/v] SDS, 0.15 M bis-Tris, and 0.1 M HCl) was loaded on gel strips. After agarose solidification, electrophoresis was performed at 10°C in a buffer (pH 8.3) containing 25 mM Trizma base, 200 mM taurine, and 0.1% (w/v) SDS, for 1 h at 35 V and for 14 h at 110 V. Ten gels (200 × 250 × 1.0 mm) were run in parallel (Isodalt system; Amersham Pharmacia Biotech). For each condition analyzed, two-dimensional gels were made in triplicate and from two independent protein extractions.

Protein Staining and Analysis of Two-Dimensional Gels

Gels were stained with silver nitrate according to a modified procedure of Blum et al. (1987) or with the GelCode blue stain reagent from Pierce (Rockford, IL), using the Hoefer Automated Gel Stainer apparatus (Amersham Pharmacia Biotech). Silver-stained gels were scanned with a scanner (JX-330; Sharp Electronics [Svenska] AB, Bromma, Sweden) equipped with Labscan, version 3.00 (Amersham Pharmacia Biotech). Image analysis was carried out with software (ImageMaster 2-D Elite, version 3.01; Amersham Pharmacia Biotech), according to the manufacturer's instructions. After spot detection and background subtraction (mode: average on boundary), two-dimensional gels were aligned, matched, and the quantitative determination of the spot volumes was performed (mode: total spot volume normalization). For each analysis, statistical data showed a high level of reproducibility between normalized spot volumes of gels produced in triplicate from the two independent protein extractions.

Protein Identification by Mass Spectrometry

Spots of interest were excised from GelCode-stained two-dimensional gels and were digested by sequence grade trypsin (Promega, Madison, WI). After digestion, the supernatant containing peptides was concentrated by batch adsorption on beads (POROS 50 R2; Roche Molecular Biochemicals, Basel) and used for MALDI-mass spectrometry analysis on a MALDI-TOF spectrometer (Reflex II; Bruker, Billerica, MA) after on-target desorption with matrix solution (Gevaert et al., 1998). Before each analysis, the instrument was externally calibrated using two synthetic

peptides spotted as near as possible to the biological sample. Proteins were identified by searching the protein databases using MASCOT (http://www.matrixscience.com). Theoretical masses and pI of identified proteins were predicted by entering the sequence at http://www.expasy.ch/tools/peptide-mass.html. To denote a protein as unambiguously identified, the following criteria were used: coverage of the protein by the matching peptides must reach a minimum of 10%, and at least four independent peptides should match within a stringent 0.001% maximum deviation of mass accuracy. In some cases, protein identities were further confirmed from PSD spectra generated from selected peptides. Search for sequence homology was carried out at http://www.arabidopsis.org/Blast.

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