

Endo- β -Mannanase Activity from Individual Tomato Endosperm Caps and Radicle Tips in Relation to Germination Rates¹

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Endo- β -mannanase is hypothesized to be a rate-limiting enzyme in endosperm weakening, which is a prerequisite for radicle emergence from tomato (*Lycopersicon esculentum* Mill.) seeds. Using a sensitive, single-seed assay, we have measured mannanase activity diffusing from excised tomato endosperm caps following treatments that alter the rate or percentage of radicle emergence. Most striking was the 100- to more than 10,000-fold range of mannanase activity detected among individual seeds of highly inbred tomato lines, which would not be detected in pooled samples. In some cases a threshold-type relationship between mannanase activity and radicle emergence was observed. However, when radicle emergence was delayed or prevented by osmoticum or abscisic acid, the initial increase in mannanase activity was unaffected or even enhanced. Partially dormant seed lots displayed a bimodal distribution of activity, with low activity apparently associated with dormant seeds in the population. Gibberellin- and abscisic acid-deficient mutant seeds exhibited a wide range of mannanase activity, consistent with their variation in hormonal sensitivity. Although the presence of mannanase activity in the endosperm cap is consistently associated with radicle emergence, it is not the sole or limiting factor under all conditions.

Even though tomato (*Lycopersicon esculentum* Mill.) is self-pollinating and cultivars are highly homozygous genetically, germination behavior varies considerably among individual seeds. We have developed a population-based hydrotime model that accurately describes germination time courses in response to environmental and hormonal factors (Bradford, 1990, 1995, 1996; Dahal and Bradford, 1990, 1994; Ni and Bradford, 1992, 1993). This model is based on the assumption that individual seeds vary in their sensitivity to these factors and particularly in the sensitivity of radicle emergence to ψ . If the ψ_b is higher (more positive) than the actual seed ψ , radicle emergence will not occur. The lower (more negative) the ψ_b for radicle emergence relative to the ambient ψ , the more rapid germination will be. The distribution of ψ_b values within the seed population therefore determines both the rate and final percentage of germination. Environmental and hormonal fac-

tors act by shifting the entire distribution of ψ_b values within the seed population to lower or higher values, consequently speeding or slowing the time to radicle emergence (Ni and Bradford, 1993; Dutta and Bradford, 1994; Bradford, 1995, 1996).

The population-based hydrotime model can quantify and describe seed germination patterns, but it does not identify the physiological or biochemical basis of ψ_b . In tomato there is considerable evidence that ψ_b is largely determined by the mechanical resistance of the micropylar tissues (endosperm cap and testa) covering the radicle tip. The force required to puncture the micropylar tissues declines prior to radicle emergence, and radicle emergence will occur at much lower ψ if the cap is removed (Groot and Karssen, 1987, 1992; Dahal and Bradford, 1990). There is an inverse correlation between the mean time to radicle emergence and the force required to puncture the endosperm cap (Karssen et al., 1989). The mechanical resistance appears to reside primarily in the endosperm rather than the testa, since the force required to penetrate the testa remained relatively constant prior to radicle emergence (Groot and Karssen, 1987), and removal of the testa alone did not eliminate genotypic effects on germination timing, whereas removal of both the endosperm and testa did (Leviatov et al., 1994). Weakening of the micropylar endosperm cap tissue appears to be a prerequisite for radicle emergence in tomato.

Groot et al. (1988) proposed that cell wall hydrolysis in the endosperm cap might be responsible for the mechanical weakening of the tissue. Since the endosperm cell walls are composed primarily of Man, Gal, and Glc, they hypothesized that endo- β -mannanase, β -mannosidase, or α -galactosidase might be involved in cell wall degradation, as in lettuce (*Lactuca sativa* L.; Bewley et al., 1983). Activities of all three enzymes increased during imbibition prior to radicle emergence, but the increase in endo- β -mannanase was the most dramatic response to GA (Groot et al., 1988) and was linearly correlated with decreasing endosperm resistance to penetration (unpublished results cited in Hilhorst and Karssen, 1992). Mannanase activity also correlated well with low-temperature germination capacity of tomato genotypes (Leviatov et al., 1995). Development of endo- β -mannanase activity is localized in the

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Abbreviations: *gib-1*, GA-deficient tomato mutant; *MM*, tomato cultivar MoneyMaker; ψ , water potential; ψ_b , threshold or base water potential; *sit^m*, ABA-deficient tomato mutant.

endosperm cap prior to radicle emergence and is reported to be inhibited by far-red light and ABA, which also block radicle emergence (Nomaguchi et al., 1995; Nonogaki and Morohashi, 1996). There are multiple isozymes of endo- β -mannanase in germinating tomato seeds (Dirk et al., 1995; Nonogaki et al., 1995; Toorop et al., 1996), but only a single isozyme appears in the endosperm cap prior to radicle emergence (Nonogaki and Morohashi, 1996). An attractive hypothesis, therefore, is that ψ_b of tomato seeds is related to the extent of weakening of the endosperm cap, which in turn is dependent primarily on the development of endo- β -mannanase activity, although other enzymes may also be involved (Sanchez et al., 1986; Leubner-Metzger et al., 1995, 1996; Black, 1996).

The hydrotime model of germination reveals that individual seeds vary widely in their ψ_b values (Bradford, 1990, 1995), and if the above hypothesis is correct, individual seeds should also vary in endo- β -mannanase activity. We developed a single-seed assay for endo- β -mannanase activity and found as much as a 1000-fold range of activity within a population of inbred tomato seeds (Still et al., 1997). Because sensitivities to ABA and GA vary more than several orders of magnitude among individual seeds (Ni and Bradford, 1993), the hypothesis further predicts that endo- β -mannanase activity in response to these hormones should also differ widely among seeds. In the present study we used the single-seed assay to determine whether the patterns of endo- β -mannanase activity within tomato seed populations are consistent with the enzyme being the rate-limiting factor controlling radicle emergence timing. Although the data do not fully support this hypothesis, the single-seed approach provides a number of novel insights into the physiology of seed populations.

MATERIALS AND METHODS

Plant Material and Seed Germination

Tomato (*Lycopersicon esculentum* Mill.) seeds of the homozygous *gib-1* and *sit^w* mutants and their wild-type isogenic parent (*MM*) were obtained from Dr. C.M. Karsen (Agricultural University, Wageningen, The Netherlands). Seeds of *gib-1* and *sit^w* were produced in a greenhouse as described previously (Ni and Bradford, 1993). Seeds of their isogenic parent line (*MM*) and of the inbred fresh market tomato line T5 were produced in the field in Davis, CA.

Seed germination conditions were essentially as described previously (Ni and Bradford, 1993). Seeds were placed on two 4.7-cm-diameter filter papers in 5-cm-diameter Petri dishes moistened with 4 mL of distilled water, (\pm)ABA (100 μ M), GA₄₊₇ (10 μ M), or PEG 8000 (−0.6 MPa) solutions and incubated at 25°C in the dark. Germinated seeds were removed frequently, and the remaining ungerminated seeds were transferred to fresh ABA, GA₄₊₇, or PEG solutions after the first 24 h and every 48 h thereafter to maintain constant concentrations. Germination was scored as radicle emergence to 1 mm.

Gel-Diffusion Assays

Activity of endo- β -mannanase released from seeds during imbibition was assayed by a gel-diffusion assay (Downie, et al., 1994; Still et al., 1997). The micropylar tip (no more than 20% of the total seed length) was cut from 45 or 60 seeds for each treatment. The radicle tip was removed from the excised endosperm and testa, and each radicle tip and cap were numbered to retain proper pairing during data analysis. Excised tissues were incubated in 20 μ L of buffer (0.1 M citric acid, 0.2 M sodium phosphate, pH 5.0) for 2 h, and a 10- μ L sample of the incubation medium was assayed by the gel-diffusion method as described previously (Still et al., 1997). Activity from the samples was standardized relative to purified endo- β -mannanase from *Aspergillus niger* (Megazyme, Sydney, Australia).

Previous results indicated that the enzyme activity diffusing from the tissues is as good as extracted activity for estimating the population distribution of mannanase activity (Still et al., 1997). Data were categorized into 0.5 log units of activity and presented as frequency diagrams of the percentage of seeds in the total population expressing a given category of enzyme activity. The mean, median, and range of activity from the individual assays at each sampling time were also calculated. The enzyme activities of radicle tips closely resembled the patterns of activity measured from endosperm caps, but in most cases, radicle activity was 1 order of magnitude less and the range of activity was not as great, compared with endosperm activity (Still et al., 1997). We show only endosperm cap activities in the figures; radicle tip activities are summarized for comparison in Table I.

RESULTS AND DISCUSSION

Theoretical Expectations

The connection between enzyme activity and radicle emergence timing must be of a statistical nature when considering the type of data to be generated from single-seed assays. Since the assays are necessarily destructive, it is not possible to know both the amount of enzyme expression prior to radicle emergence and the time that emergence would have occurred if the seed had not been sampled. It is possible, however, to make some predictions about the types of population distributions of activity that might be expected and how they could be used to test causal connections.

Three possible scenarios are illustrated as frequency diagrams (Fig. 1). The first can be called the "enzyme threshold model," in which enzyme activity increases in all seeds with time after imbibition, and radicle emergence occurs when the activity exceeds a specific threshold amount (Fig. 1, A–D). Variation in germination timing is due to the variation in how rapidly individual seeds increase their enzyme activity to the threshold amount. If only the remaining ungerminated seeds are sampled through a time course (Fig. 1, solid bars), their enzyme activities would approach the threshold. Seeds with activities exceeding the threshold would not be present, because they would have already completed germination and would be excluded

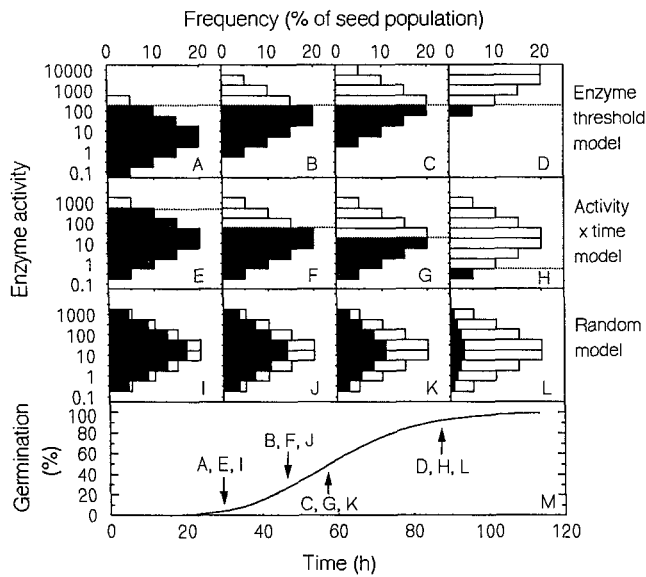


Figure 1. Alternative models of how frequency distributions of enzyme activities within seed populations might change during a germination time course. M, A typical time course of radicle emergence. The points marked by the arrows indicate sampling times that might result in the corresponding enzyme frequency distributions in the indicated panels above (solid bars are ungerminated seeds; open bars are seeds that have completed germination and would not be sampled for activity). A to D, An enzyme threshold model, in which completion of germination occurs after enzyme activity has increased to a threshold amount (dotted line). E to H, An activity \times time model, in which the activity per seed remains constant but germination is completed after a longer time when the activity is lower. The product of activity \times time is the same for all seeds, but the apparent threshold (dotted line) decreases with time. I to L, A random model, in which completion of germination occurs randomly in the population with respect to the enzyme activity measured. This pattern would indicate that the enzyme is not closely associated with the timing of radicle emergence, because seeds in the population complete germination regardless of the enzyme activity.

from the assay (Fig. 1, open bars). Endo- β -1,3-glucanase activity was expressed in a manner somewhat consistent with this model in response to ABA (Leubner-Metzger et al., 1995).

A second possibility is that individual seeds inherently have the capacity to express only a particular amount of enzyme activity that is established following imbibition. The time to radicle emergence might then be inversely proportional to the enzyme activity, i.e. activity multiplied by time is a constant. This could be termed the “activity \times time” model. In this case the apparent threshold activity allowing germination would decrease with time as individual seeds met their activity \times time requirement and completed germination (Fig. 1, E–H). Sampling only ungerminated seeds allows discrimination between these two models, because in one case the enzyme activity of the ungerminated seed fraction increases over time, whereas in the other it decreases (Fig. 1, A–D versus E–H, solid bars).

A third possibility is that germination occurs randomly in the population regardless of the enzyme activity, or a “random model” (Fig. 1, I–L). This would suggest that,

although the enzyme is present in germinating seeds, its activity is not intimately connected with the timing of radicle emergence. Other factors (other enzymes, anatomical variation, etc.) would be controlling radicle emergence in this case. Other scenarios are possible, but these examples illustrate how population-based data can be used to test specific hypotheses regarding the involvement of a given enzyme in controlling germination.

T5 Seeds Germinating in Water

Seeds of an inbred tomato line (T5) were allowed to imbibe in water, and mannanase activity diffusing from individual endosperm caps was assayed during the germination time course. After 12 h of imbibition mannanase activity ranged from less than 1 to more than 100 pmol min⁻¹ cap⁻¹ (Fig. 2A; Table I). A bimodal distribution was apparent, with about 70% of the population having activity less than 10 pmol min⁻¹ cap⁻¹. Median mannanase activity increased with imbibition time as germination occurred, but the range of mannanase activity among endosperm caps varied by 2.5 (Fig. 2, A, C, and E) to 3.5 (Fig. 2, B and D) log units (Table I). Because mannanase activity of most seeds gradually increased over time, the pattern most closely matched the enzyme threshold model in which mannanase activity above a threshold coincides with radicle emergence (Fig. 1, A–D). However, it is not possible to determine a single mannanase activity threshold that precisely matches the fraction germinating for each sampling period. Although mannanase activity generally increased over time for many seeds in the population, there was always a wide range of activities present at all sampling times.

According to the enzyme threshold model, all seeds that are on the verge of radicle emergence should express at or near the threshold enzyme activity. We therefore allowed

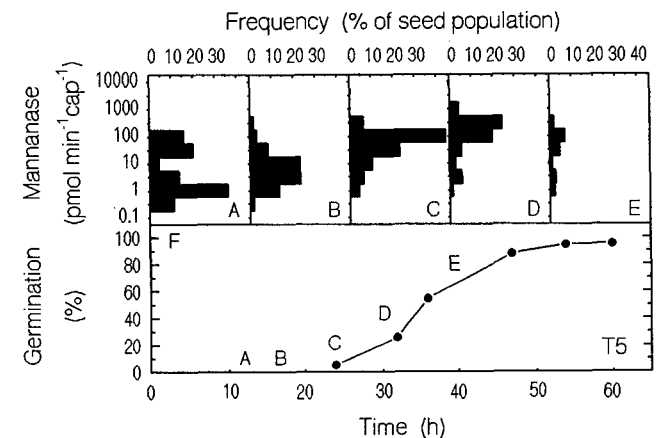


Figure 2. Frequency distributions of endo- β -mannanase activity of endosperm caps excised from T5 seeds that were allowed to imbibe in water and the corresponding radicle emergence time course (F). Panels illustrating mannanase activity correspond to sampling times as indicated along the time course: 12 (A), 16 (B), 24 (C), 32 (D), and 40 (E) h after imbibition. Only the remaining ungerminated seeds at each time were sampled, and the percentages are based on the total seed population.

Table 1. Statistical parameters for endo- β -mannanase activity from tomato endosperm caps and radicle tips under various germination conditions

The mean, median, and range of activity were calculated from the individual assays of *n* endosperm caps (Cap) and radicle tips (Tip) for each sampling time. The corresponding figure showing the frequency distribution for the endosperm cap activities is indicated.

Genotype	Treatment	Time	<i>n</i>	Figure	Tissue	Mean	Median	Range
		<i>h</i>	<i>pmol min⁻¹ cap⁻¹ or tip⁻¹</i>					
T5	Water	12	45	2A	Cap	41.6	2.94	279
					Tip	22.7	1.66	195
		16	60	2B	Cap	32.7	8.6	620
					Tip	6.87	1.45	40.6
		24	45	2C	Cap	121	97.1	490
					Tip	10.3	5.5	58.3
		32	45	2D	Cap	34.5	284	1,350
					Tip	11.7	9.4	54.5
		40	60	2E	Cap	138	76.2	903
					Tip	6.6	3.5	31
T5	Near germination	28	15	3A	Cap	465	433	1,300
					Tip	12.1	8.5	36
		31	45	3B	Cap	656	568	2,010
					Tip	6.2	3.7	57
		34	45	3C	Cap	542	422	1,940
T5	-0.6 MPa				Tip	5.5	4.0	27
		46	30	3D	Cap	200	181	418
					Tip	15.5	3.5	308
		20	45	4A	Cap	946	663	246
					Tip	868	725	2,190
T5	-0.6 MPa	118	45	4B	Cap	3,840	2,631	12,510
					Tip	1,026	982	2,360
		222	45	4C	Cap	678	467	3,820
					Tip	150	144	379
		360	46	4D	Cap	194	80	2,060
T5	100 μ M ABA				Tip	86	50	649
		23	60	5A	Cap	17.4	5.4	177
					Tip	47.8	9.5	401
		46	60	5B	Cap	1,342	904	6,690
					Tip	19	11.5	116
T5	100 μ M ABA	70	60	5C	Cap	558	250	4,970
					Tip	12.6	7.5	69.1
		94	60	5D	Cap	109	37	686
					Tip	6	3	70
		120	60	5E	Cap	6	2	49
MM	Water				Tip	2	0.9	25
		24	45	6A	Cap	42	5	504
					Tip	4	3	21
		48	60	6B	Cap	87	37	1,040
					Tip	10	3	116
MM	Water	72	45	6C	Cap	91	67	409
					Tip	1	1	6
		96	45	6D	Cap	51	21	302
					Tip	3	2	14
		120	45	6E	Cap	24	1	628
gib-1	10 μ M GA ₄₊₇				Tip	1	0.4	5
		12	45	7A	Cap	36	3	811
					Tip	2	2	9
		24	45	7B	Cap	53	4	2,170
					Tip	5	2	56
gib-1	10 μ M GA ₄₊₇	48	60	7C	Cap	753	64	14,850
					Tip	760	47	21,850
		96	45	7D	Cap	13	5	108
					Tip	7	5	45
		144	45	7E	Cap	40	1	809
sit ^w	Water				Tip	2	1	9
		6	45	8A	Cap	808	10	27,240
					Tip	3	0.3	35
		24	45	8B	Cap	14	5	110
					Tip	36	11	495
sit ^w	Water	48	45	8C	Cap	63	9	594
					Tip	4	0.5	89

T5 seeds to imbibe in water for 28, 31, 34, and 46 h and selected only seeds in which the radicle was about to protrude through the micropylar tip. These seeds were easily identified by the outline of the radicle at the micropylar end. In addition, the pressure from the razor blade applied during excision of the micropylar tip was enough to cause radicle protrusion, which was never observed in seeds unless emergence was imminent. The frequency distribution of mannanase activity in this subpopulation of seeds was similar at each sampling time and was centered near the high end of the activity range (Fig. 3; Table I). The cumulative frequency was equal to 100% at each sampling time because the sample constituted the entire population of seeds in which radicle emergence was incipient at that time. These results are consistent with the hypothesis that T5 seeds that were allowed to imbibe in water follow the enzyme threshold model, but they do not prove that mannanase is actually responsible for radicle emergence.

T5 Seeds Germinating at Reduced ψ

Reduced ψ delays germination and has been reported to both increase (Karssen et al., 1989; Nonogaki et al., 1992; P. Dahal, D.J. Nevins, and K.J. Bradford, unpublished data) and decrease (Hilhorst and Downie, 1996) mannanase activity in the endosperm cap. Imbibition of T5 seeds in -0.6 MPa PEG inhibited final radicle emergence percentage and markedly decreased the emergence rate, compared with seeds that were allowed to imbibe in water (Fig. 4E). Mannanase activity, however, was high in seeds as early as 24 h after imbibition, long before the first seeds completed germination (Fig. 4A). Median activity increased even further before any radicle emergence occurred, with some seeds having very high activity (Fig. 4B; Table I). Mannanase activity varied over a wide range in seeds that did not complete radicle emergence at this ψ (Fig. 4D). A similar

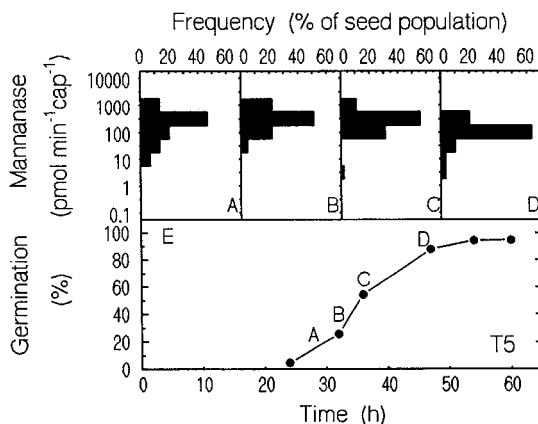


Figure 3. Frequency distributions of endo- β -mannanase activity of endosperm caps excised from T5 seeds that were allowed to imbibe in water and in which radicle emergence was imminent. Panels illustrating mannanase activity correspond to enzyme activity 28 (A), 31 (B), 34 (C), and 46 (D) h after imbibition. At each time, only seeds on the verge of radicle emergence were selected. The percentages total 100% at each time, representing the entire population of seeds in which radicle emergence was imminent.

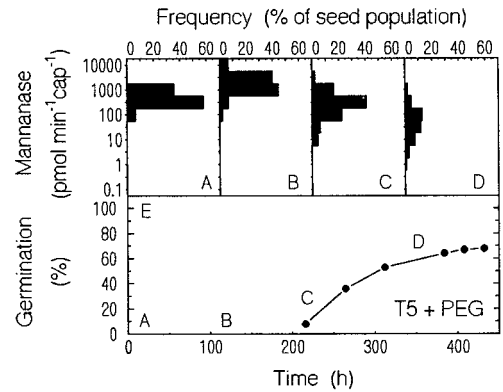


Figure 4. Frequency distributions of endo- β -mannanase activity of endosperm caps excised from T5 seeds that were allowed to imbibe in -0.6 MPa PEG and the corresponding radicle emergence time course (E). Panels illustrating mannanase activity correspond to sampling times as indicated along the time course: 20 (A), 118 (B), 222 (C), and 360 (D) h after imbibition. Only the remaining ungerminated seeds at each time were sampled, and the percentages are based on the total seed population.

pattern of increasing and then decreasing activity was detected in extracts from pooled endosperm cap samples from seeds incubated at reduced ψ (P. Dahal, D.J. Nevins, and K.J. Bradford, unpublished data).

The occurrence of high mannanase activity for a long period before radicle emergence argues against mannanase being the sole factor determining emergence rate. Reduced ψ will also lower embryo turgor, but previous experiments have demonstrated that if the endosperm cap is removed radicle emergence is more rapid at -0.6 MPa than it is in water with an intact endosperm cap (Dahal and Bradford, 1990). Thus, embryo growth potential should not be limiting radicle emergence if the endosperm is weakened to the same extent that it is in water (Nomaguchi et al., 1995; Toorop et al., 1996). If endosperm weakening was occurring in proportion to the amount of mannanase activity, as is implied by the experiments in water described above, there should be sufficient enzyme for weakening and radicle emergence to occur at -0.6 MPa far sooner than it actually does. It also appears that even seeds that will eventually be prevented from completing radicle emergence at -0.6 MPa (i.e. their $\psi_b > -0.6$ MPa) express high mannanase activity early in imbibition, since all seeds displayed activity at or near the apparent activity threshold (Fig. 4B). It is also apparent that reduced ψ does not delay radicle emergence via a direct inhibition of mannanase activity, as had been hypothesized (Ni and Bradford, 1993). Rather, reduced ψ appears to prevent additional weakening processes that have been proposed on the basis of mechanical resistance measurements (Haigh, 1988; Karssen et al., 1989).

T5 Seeds Germinating in ABA

ABA delayed and inhibited radicle emergence from tomato seeds (Fig. 5F). Similar to the effect of reduced ψ , however, median mannanase activity increased sharply

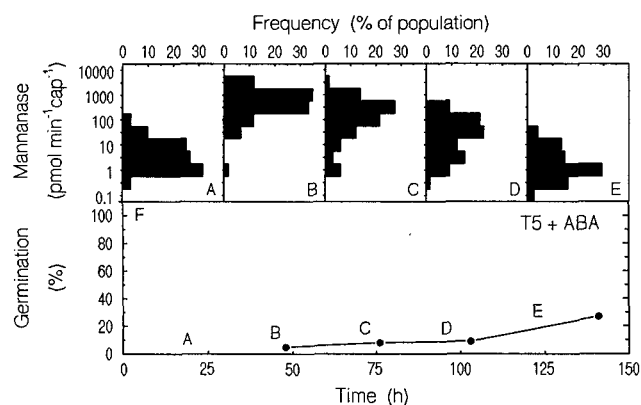


Figure 5. Frequency distributions of endo- β -mannanase activity of endosperm caps excised from T5 seeds that were allowed to imbibe in 100 μM ABA and the corresponding radicle emergence time course (F). Panels illustrating mannanase activity correspond to sampling times as indicated along the time course: 23 (A), 46 (B), 70 (C), 94 (D), and 120 (E) h after imbibition. Only the remaining ungerminated seeds at each time were sampled, and the percentages are based on the total seed population.

and then declined gradually before a significant fraction of the seed population had completed germination (Fig. 5, A–E; Table I). The overall pattern of enzyme activity was similar to that of extracted mannanase from pooled endosperm cap samples (P. Dahal, D.J. Nevins, and K.J. Bradford, unpublished data). However, pooled samples did not reveal the very wide range of activity that exists among seeds, which was at least 100-fold, and often more than 1000-fold among individual endosperm caps (Table I). The increase and then decrease in mannanase activity is suggestive of a feedback inhibition process in which some factor other than mannanase limits radicle emergence. Similar patterns have been recorded for respiration when seeds are allowed to imbibe under conditions in which germination will not be completed (Powell et al., 1984; Dahal et al., 1996).

The high mannanase activity in ABA-treated tomato endosperm caps found here and previously in our laboratory (P. Dahal, D.J. Nevins, and K.J. Bradford, unpublished data) and by others (Toorop et al., 1996) is in contrast to the absence of such activity reported by Nomaguchi et al. (1995). The latter experiment included only a single time point at 72 h of imbibition; therefore, it is possible that by that time mannanase activity had already declined below the sensitivity of the assay used. On the other hand, ABA inhibits development of mannanase synthesis or activity in lettuce (*Dulson et al., 1988; Dutta et al., 1996*) and fenugreek (*Trigonella foenum-graecum* L.; Malek and Bewley, 1991) endosperm. Groot and Karssen (1992) showed that ABA inhibits mechanical weakening of the tomato endosperm cap, but we found no inhibition of mannanase activity at a time when seeds that were allowed to imbibe in water would have completed radicle emergence. It is possible that endosperm weakening occurred but that embryo growth was inhibited by ABA (Schopfer and Plachy, 1985). However, initial radicle protrusion is not prevented by ABA in seeds from which the endosperm cap is re-

moved (Nomaguchi et al., 1995; Toorop et al., 1996), and other data also indicate that ABA action is primarily on the endosperm (Groot and Karssen, 1992; Hilhorst and Downie, 1996). Thus, although mannanase may have a role in endosperm weakening, its presence is not always accompanied by endosperm weakening and radicle emergence.

MM, *gib-1*, and *sit^{sw}* Seeds

Under our production conditions, about 30% of MM tomato seeds were dormant, since only 70% of the seeds completed germination in water (Fig. 6F). A wide range of mannanase activities was observed among endosperm caps by 24 h, approximately 24 h before the first seeds germinated (Fig. 6A). By 48 h a bimodal distribution of mannanase activity was evident, and the two subpopulations became even more distinct by 72 h (Fig. 6, B and C). After 72 h approximately 25% of the population, presumably the dormant seeds, had mannanase activities of 1 $\text{pmol min}^{-1} \text{cap}^{-1}$ or less. At longer imbibition times the seeds with higher mannanase activity apparently completed germination and were no longer in the sample population, because most of the (dormant) seeds remaining at 120 h had low activities, although there was still a wide variation among seeds. Imbibition of MM seeds in 100 μM GA₄₊₇ will stimulate all seeds to germinate rapidly (Ni and Bradford, 1993). When assayed for mannanase activity after 48 h of imbibition on 100 μM GA₄₊₇, the population had a median of 316 $\text{pmol min}^{-1} \text{cap}^{-1}$, a mean of 366 $\text{pmol min}^{-1} \text{cap}^{-1}$, and a range of 1280 $\text{pmol min}^{-1} \text{cap}^{-1}$ (data not shown). This activity is much higher than for the seeds that were allowed to imbibe on water (Table I), and the bimodal distribution (Fig. 6B) was no longer evident (data not shown). These data are consistent with the enzyme threshold model (Fig. 1, A–D), at least for nondormant or GA-treated seeds.

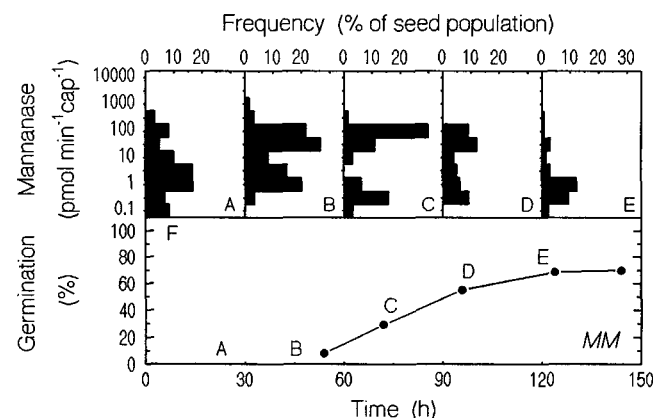


Figure 6. Frequency distributions of endo- β -mannanase activity of endosperm caps excised from MM seeds that were allowed to imbibe in water and the corresponding radicle emergence time course (F). Panels illustrating mannanase activity correspond to sampling times as indicated along the time course: 24 (A), 48 (B), 72 (C), 96 (D), and 120 (E) h after imbibition. Only the remaining ungerminated seeds at each time were sampled, and the percentages are based on the total seed population.

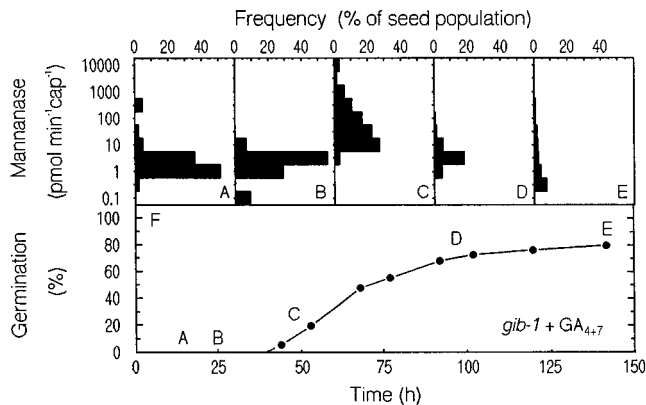


Figure 7. Frequency distributions of endo- β -mannanase activity of endosperm caps excised from *gib-1* seeds that were allowed to imbibe in $10 \mu\text{M}$ GA_{4+7} and the corresponding radicle emergence time course (F). Panels illustrating mannanase activity correspond to sampling times as indicated along the time course: 12 (A), 24 (B), 48 (C), 96 (D), and 144 (E) h after imbibition. Only the remaining ungerminated seeds at each time were sampled, and the percentages are based on the total seed population.

The value of single-seed assays is clearly demonstrated by these data on a partially dormant seed lot. The clear separation of the seed population into two subfractions, one with high and one with low activity (Fig. 6, B and C), would be impossible with pooled samples. Activity in pooled samples would reflect the means (Table I), which show only an increasing and then decreasing pattern, rather than the bimodal distribution evident from the individual assays. Using the single-seed approach, we can identify, prior to radicle emergence, those seeds with low mannanase activity, and we can anticipate with some confidence that they would largely represent the dormant fraction of the population. This does not automatically imply that low mannanase activity limits germination of dormant seeds, but it does indicate that mannanase (and presumably other required enzyme activities) does not increase to the same extent as in nondormant seeds.

The *gib-1* mutant is blocked in GA synthesis and radicle emergence of its seeds is completely dependent on the addition of GA (Groot and Karssen, 1987). When seeds were allowed to imbibe in water, radicle emergence did not occur, and mannanase activity was never detected from endosperm caps that were allowed to imbibe for up to 168 h (data not shown). The addition of $10 \mu\text{M}$ GA_{4+7} caused radicle emergence from *gib-1* seeds with a time course similar to that of the wild-type isogenic parent *MM* seeds (Fig. 7F; Groot and Karssen, 1987; Ni and Bradford, 1993). However, mannanase activity of *gib-1* seeds that were allowed to imbibe in $10 \mu\text{M}$ GA_{4+7} remained well below that present in germinating fractions of *MM* seeds (compare Fig. 6, A and B, with Fig. 7, A and B) but at least 10- to 100-fold greater than that in *gib-1* seeds in the absence of GA_{4+7} (always below detection, or $<0.5 \text{ pmol min}^{-1}$). As radicle emergence was beginning to occur, a wide array of mannanase activities appeared (Fig. 7C; Table I), and seeds unable to complete germination at this GA_{4+7} concentration eventually exhibited relatively low

activities (Fig. 7, D and E). At intermediate GA_{4+7} concentrations that do not induce all seeds to germinate, there appeared to be a variable response among seeds in mannanase expression, and a relatively unresponsive seed fraction may never elevate mannanase (and presumably other required enzymes) to the extent required for endosperm weakening. This highly variable biochemical response is in agreement with the wide GA_{4+7} sensitivity distribution characterized previously (Ni and Bradford, 1993).

Leviatov et al. (1995) found that treatment of tomato seeds with a bacterial endomannanase speeded up germination rates by about 10%. If GA acts primarily through the induction of endo- β -mannanase, then direct exposure of *gib-1* seeds to mannanase should also result in endosperm weakening and radicle emergence. *gib-1* seeds with the testae removed were incubated in endo- β -mannanase ($1000 \text{ pmol min}^{-1}$) purified from *A. niger*. Direct external exposure to a high activity of fungal mannanase did not induce radicle emergence in *gib-1* seeds (data not shown). There are many possible explanations for this lack of effectiveness, including inability of the enzyme to penetrate to specific cell wall sites, a requirement for additional cooperative enzymes, and differing structural requirements for activity between the fungal and tomato enzymes, even though both will hydrolyze locust bean galactomannan. Nonetheless, this simple experiment suggests that the presence of high mannanase activity alone is not sufficient to allow radicle penetration through the endosperm cap.

ABA-deficient *sit^w* mutant seeds germinate more rapidly than do seeds of their parental line, *MM* (Fig. 8D; Groot and Karssen, 1992; Ni and Bradford, 1993). Endosperm caps isolated after 6 h of imbibition, well before any seeds had completed germination, showed a remarkable range of

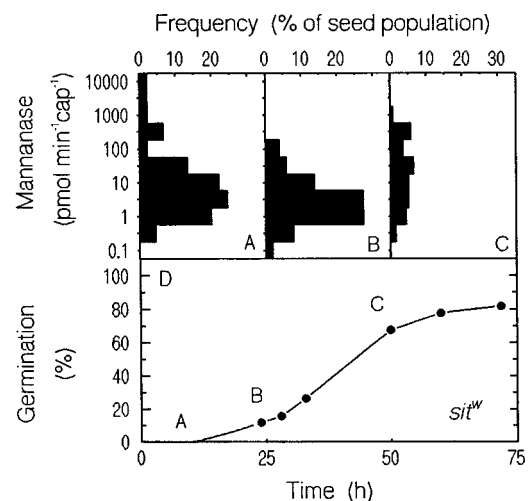


Figure 8. Frequency distributions of endo- β -mannanase activity of endosperm caps excised from *sit^w* seeds that were allowed to imbibe in water and the corresponding radicle emergence time course (D). Panels illustrating mannanase activity correspond to sampling times as indicated along the time course: 6 (A), 24 (B), and 48 (C) h after imbibition. Only the remaining ungerminated seeds at each time were sampled, and the percentages are based on the total seed population.

more than 27,000 pmol min⁻¹ cap⁻¹ (Fig. 8A; Table I). Because of the logarithmic range in mannanase activity, only three seeds of 45 with exceptionally high activity accounted for 93% of the total observed at this sampling point and shifted the mean 80-fold higher than the median (Table I). Those seeds apparently were among the first to germinate, because the remainder of the activity distribution was relatively unchanged thereafter, except for the decrease in total number of seeds as additional seeds germinated. It is interesting that mean mannanase activity in radicle tips was 2.5-fold greater than that of endosperm caps at the 24 h sampling time (Table I). This was one of only two instances among all the experiments reported in which activity in the radicle tips was equal to or exceeded that of the endosperm caps. The pattern of mannanase activity from endosperm caps most closely resembles the random model (Fig. 1, I–L), in which seeds germinate throughout the population without regard to their mannanase activity, and the last remaining ungerminated seeds exhibit a wide range of activity.

Hilhorst and Downie (1996) found that endo- β -mannanase activity was approximately 50% greater in *sit*^w seeds than in *MM* seeds. This is consistent with our finding of high mean activity prior to radicle emergence (Fig. 8A; Table I) but should be interpreted with caution considering the wide range of activity present in both *sit*^w and *MM* seeds (Figs. 6 and 8) and the influence that relatively few seeds with exceptional activity can have in pooled samples. In addition, because of low ABA content during development, *sit*^w seeds are prone to vivipary and exhibit cell cycle activity and free space in the dry seed characteristic of seeds that have progressed toward germination prior to dehydration (Argerich and Bradford, 1989; Groot and Karsen, 1992; Liu et al., 1993, 1994; Hilhorst and Downie, 1996). Thus, they are in some respects similar to osmotically treated (primed) seeds that also exhibit high mannanase activity (Fig. 4; Karssen et al., 1989; but see contrasting results by Hilhorst and Downie, 1996) and undergo partial endosperm weakening. The testa may also play a more important role in regulating germination in *sit*^w seeds than it does in wild-type seeds (Hilhorst and Downie, 1996). In subsequent work we have found that *sit*^w seed lots can have variable characteristics depending, for example, on fruit maturity at harvest, and that there is a high degree of variability among individual seeds (B. Downie, S. Gurusinghe, and K. Bradford, unpublished results). Single-seed assays are particularly appropriate for studying such seed lots to avoid the disproportionate effects that a few highly expressing individuals can have on pooled samples.

CONCLUSIONS

A single-seed assay was utilized to characterize expression of endo- β -mannanase activity in tomato endosperm caps and radicle tips prior to radicle emergence. If mannanase activity is the rate-limiting factor in endosperm weakening and radicle penetration, then there should be a consistent relationship between activity and radicle emergence, since emergence rate and percentage are varied by

environmental or hormonal manipulations. Mannanase activity was consistently present in hydrated seeds (except for *gib-1* seeds without added GA), and its activity was uniformly high in wild-type seeds just prior to radicle emergence. During incubation in osmoticum or ABA, however, high mannanase activity accumulated without radicle protrusion, even though embryo growth potential should not be limiting in either case. In some cases mannanase activity appeared to conform to a threshold model (e.g. T5 or *MM* seeds in water), whereas in other cases no clear direct relationship between mannanase activity and radicle emergence was evident (wild-type seeds in osmoticum or ABA, *gib-1* seeds in GA, *sit*^w seeds in water). Mannanase appears to be among the factors that are related to completion of germination, because low mannanase activity early in imbibition was a good marker of subsequent dormancy in *MM* seeds. However, this does not prove that mannanase activity is solely responsible for endosperm weakening, because other enzymes might also exhibit a similar regulatory pattern. In agreement with Toorop et al. (1996), we conclude that, although endo- β -mannanase activity consistently is (or has been) present in the endosperm cap when tomato radicles emerge, it does not appear to be the sole or rate-limiting enzyme in this process.

A significant component of this work is the demonstration that single-seed assays can be used to investigate the biochemical behavior of seed populations. A remarkable and unexpected variation in enzyme activities was present among individual seeds, often exhibiting a range of more than 1,000-fold and occasionally more than 10,000-fold. Variation of this magnitude would never be detected using pooled assays, and, as demonstrated here, it is possible for most of the activity in a pooled assay to be contributed by only a small fraction of the sampled population. Correlations between pooled activity measurements and population behavior therefore must be viewed with caution. However, the extreme diversity of enzyme activity among seeds is consistent with the wide variation in seed sensitivity to ψ and hormones predicted by the hydrotime and hormone-time models (Ni and Bradford, 1993; Bradford, 1995, 1996). Because highly inbred tomato lines were used in this study, the variation in mannanase activity apparently represents "physiological segregation" of the seed population rather than genetic segregation. Anatomical variation (e.g. endosperm or testa thickness) might also contribute to a lack of simple correlation between enzyme activity and speed of emergence, and this is currently under investigation. It is critical when attempting to assign causality to a biochemical response mechanism to know whether all seeds (or cells) are responding uniformly or whether stimulus/response correlations are due to variable sensitivity among individuals and recruitment of additional responders as stimuli increase (Bradford and Trewavas, 1994). This question can only be addressed by designing experiments that maintain the population level information (Bradford, 1996). Reporter genes, tissue prints, and in situ technologies, along with analytical models appropriate for variable populations, will be valuable tools in pursuing these questions.

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