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The distribution of ATP within tomato (*Lycopersicon esculentum* Mill.) embryos correlates with germination whereas total ATP concentration does not

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Abstract

The distribution of ATP in tomato seeds was visualized by monitoring the luminescence of frozen sections on top of a gel containing all the components of the luciferase reaction, but excluding ATP. ATP was imaged in germinating tomato seeds at intervals of 3, 6, 17, 24 and 48 h and in seeds with primary or secondary dormancy. ATP was present mainly in the embryo and concentrated in the radicle tip towards the completion of germination. In contrast to germinating seeds, ATP was distributed more evenly in dormant seeds. For germination, the ratio of ATP concentration in the radicle tip versus cotyledons was decisive, rather than the absolute concentration.

Keywords: *Lycopersicon esculentum*, tomato, ATP, luciferase, germination, dormancy

Introduction

ATP is the main energy source for biological processes, including seed germination. In the quiescent dry seed, the adenosine pool is mainly composed of AMP and ADP (Bewley and Black, 1994). ATP is synthesized rapidly upon water uptake and resumption of metabolic activity in the seed. ATP is essential for germination and is used in anabolic processes such as RNA and protein synthesis (Coolbear *et al*., 1990; De Castro *et al*., 1995).

Dormant seeds show active gene expression, protein synthesis and ATP accumulation (Goldmark *et al*., 1992; Li and Foley, 1995). This clearly indicates that dormancy is an actively maintained physiological state in seeds. Dormancy may be considered as a mechanism to survive prolonged periods of unfavourable conditions and, hence, energy expenditure in the dormant state is expected to be low. The extent to which dormant seeds synthesize ATP has been the subject of several studies. Changes in dormancy do not necessarily coincide with changes in respiratory activity or ATP synthesis (Derkx *et al*., 1994). Dormancy is often observed in seeds of wild species, but despite extensive breeding, dormancy may also occur in cultivated species, such as tomato (Benjamin, 1990; Still *et al*., 1997). Obviously, mechanisms that control seed dormancy are still present in cultivated species.

Many authors have adopted the classification of primary and secondary dormancy (Karssen, 1982). Seeds may acquire primary dormancy towards the end of development and the start of desiccation on the mother plant. Seeds that disperse from the mother plant can either be non-dormant or primary dormant. Freshly harvested seed batches of tomato may contain a proportion of seeds in a state of primary dormancy (Still and Bradford, 1997; De Castro, 1998). Such a batch is, therefore, a mixture of dormant and non-dormant seeds. Primary dormancy disappears within months of after-ripening in the dry state, or can be relieved by cold stratification of seeds in the imbibed state. If non-dormant imbibed seeds receive external signals that inhibit germination, they may acquire secondary dormancy. Secondary dormancy can be induced in tomato by far-red light irradiation (De Castro *et al*., 2001). Cold stratification is also efficient for relieving seeds from secondary dormancy.

The firefly (*Photinus pyralis*) luciferase–luciferin system is a useful tool in plant and animal science (Aflalo, 1991). Luciferase catalyses the decarboxylation of luciferin. Thereby, it consumes ATP and oxygen and generates a photon (565 nm). The number of emitted photons under defined assay conditions is a direct measurement of the number of ATP molecules that were converted to AMP and PP_i. The luciferase–luciferin system has been used in plant and animal research in three ways: (1) it has been used to measure

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ATP in extracts of plant tissues; (2) it has been used as a reporter gene for studying expression of native genes in plants and animals (Van der Krol and Chua, 1991; Van Leeuwen *et al*., 2000); and (3) Walenta *et al*. (1990) have developed a method based on the luciferase–luciferin system to resolve ATP distributions spatially in cryosections of tumour spheroids. In the present research, the luciferase–luciferin system was utilized to resolve ATP distributions spatially in cryosections of dormant and non-dormant seeds of tomato.

Materials and methods

Seed material

Wild-type and transgenic 35S::Luciferase (cv. Moneymaker) seeds were used for ATP imaging. Transgenic seeds were obtained from a homozygous transformant line (T_5) containing two copies of a reporter gene construct, consisting of the cauliflower mosaic virus (CaMV) promotor (−348 to +8 sequence) fused to the original luciferase coding sequence (Spoelstra *et al*., 1999). Wild-type tomatoes were harvested in 1991 and transgenic tomatoes in 1999. Seeds were extracted from ripe tomatoes and stirred in 1% (v/v) HCl for 2 h to remove locular tissue, dried and stored at 5°C. For germination experiments, 25 seeds were imbibed in 1 ml of distilled water in 50 mm Petri dishes on one layer of filter paper (Schleicher and Schuell no. 595). Seeds were imbibed in water for 3, 6, 17, 24 or 48 h. During imbibition seeds were kept in the dark at 25°C.

Induction and relief of secondary dormancy

Secondary dormancy was induced in wild-type seeds by far-red ($\lambda > 730$ nm) irradiation for 5 min at hourly intervals during the first 24 h of imbibition at 21°C and subsequent incubation in the dark at 25°C for 5 days (De Castro *et al*., 2001). During this period less than 3% of the seeds germinated. To break secondary dormancy, seeds were given a cold treatment of 3 d at 4°C, after which the seeds were transferred to 25°C in the dark, and left to germinate for 24 h.

Measurement of luciferase expression in 35S::luciferase seeds

Twenty-five transgenic seeds containing the CaMV 35S-luciferase construct were imbibed in 2.5 ml of a 0.1 mM luciferin solution (Molecular Probes, Eugene, OR, USA) in 5 cm wide plastic containers on three layers of filter paper. Photons emitted by seeds were resolved spatially using a liquid nitrogen cooled CCD camera (Versarray-512B, Roper Scientific, Princeton Instruments, Trenton, NJ, USA) operated via Metamorph 4.1 (Universal Imaging Corp., Downingtown, PA, USA) software. At 48 h of imbibition, seeds with primary dormancy were selected, based on the absence of luciferase activity.

Imaging of ATP distribution in cryosections of tomato seeds

Visualization of ATP is based upon the reaction of firefly luciferase and luciferin with ATP, O_2 and Mg^{2+}. At a pH of 7–8, the reaction generates oxyluciferin and photons at a wavelength of 562 nm with a quantum efficiency of 0.88 (Gould and Subramani, 1988). The number of emitted photons is related directly to the number of ATP molecules converted to AMP and PPi, with a stoichiometry of 1:1 (DeLuca and McElroy, 1974). Imbibing seeds were frozen in liquid nitrogen, and 20 µm median sections from ten seeds per imbibition interval were cut on a cryostat (Microm CR50 H, Bio-Med, Heidelberg, Germany) at −20°C with a steel knife. Sections were collected on glass slides at −20°C with a frozen film of 50 µl of buffered gel containing luciferase and luciferin. This gel (see also Walenta *et al*., 1990) consisted of 6% gelatin (w/v), 300 mM glycerol, 200 mM Hepes (pH 7.75), 100 mM disodium hydrogen arsenate, 3% polyvinylpyrolidone (M_r 44,000; w/v), 10 mM $MgCl_2$, 1 mM luciferin and 2 U/l luciferase (Boehringer, Mannheim, Germany). The glass slides with the cryosections were kept frozen at −20°C until measurement. At the beginning of the measurement, cryosections thawed within a few seconds at 25°C. ATP molecules from the thawed cryosection reacted with the luciferin and luciferase in the gel. Light emission from this bioluminescence reaction was captured by on-chip integration for 3 min with an intensified 16-bit CCD camera and image intensifier, with a 50 mm lens (Nikon, Melville, NJ, USA) and 70 mm c-mount extension tubes (Argus-50/2D-luminometer, Hamamatsu Photonics, Japan), in slice mode at a gain of 9.8. Although the reaction continued for a period of about 20 min, emitted photons were only integrated during the first 3 min. Prolonged measurements led to a distorted image of ATP distribution, due to diffusion of ATP through the gel.

Calibration and data analysis

The pixel intensities in 16-bit monochrome images of the bioluminescence reaction were measured using Metamorph 4.0 (Universal Imaging Corp.). The intensity of the bioluminescence reaction was calibrated to ATP concentrations, using frozen filter-paper discs (diameter 2.5 mm) with 1 µl of ATP solutions ranging from 25 to 450 mM (Fig. 1). The discs were placed on top of the reaction gel, and photon emission was integrated during the first 3 min. The bioluminescence intensity was quantified in the radicle and whole sections.

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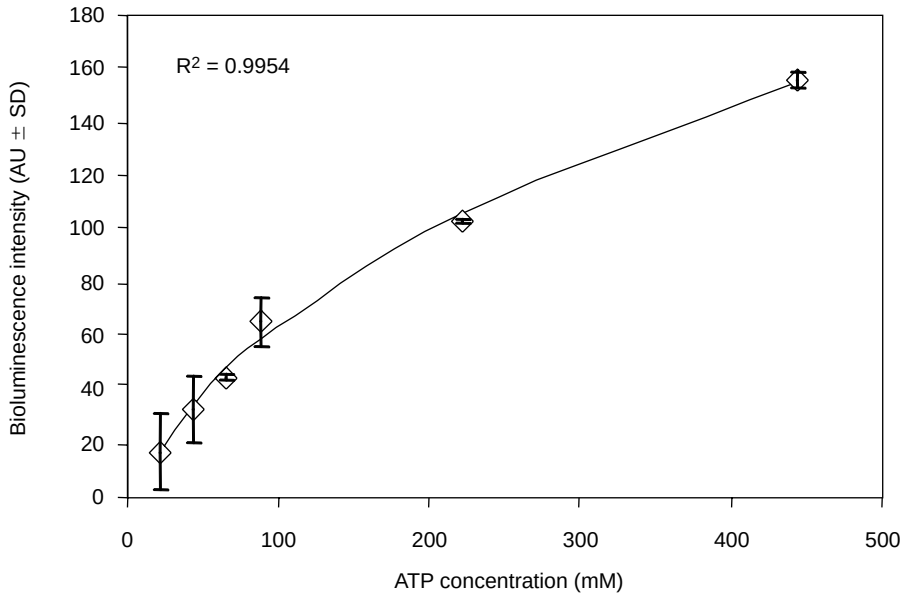

Figure 1. Calibration curve of bioluminescence intensity expressed in arbitrary units (AU) plotted against the apparent ATP concentration expressed in mM, obtained by measuring the luminescence of filter-paper discs containing different concentrations of ATP. Bars indicate standard deviations.

Statistical analysis of the data was performed via a paired-sample Student's *t*-test (comparison of ATP distribution differences within seeds) or independent-sample Student's *t*-test (comparison of ATP distribution differences between seeds of different imbibition intervals) at a confidence level of 95% with the SPSS 7.5.2 program (SPSS Inc., Chicago, IL, USA).

Extraction of ATP

The extraction of ATP was performed with a trichloroacetic acid (TCA) extraction procedure modified from Saglio and Pradet (1980). Triplicates of ten wild-type seeds were frozen in liquid nitrogen. The seeds were ground with two iron balls (diameter 6 mm) in a 2.2 ml Eppendorf tube using a Mikro-Dismembrator U (Braun Biotech International GmbH, Germany), at 1600 rpm for 3 min. To the ground seeds, 100 µl of a 0.6 M TCA solution in diethyl ether at –20°C was added. Samples were then homogenized in 2 × 250 µl and 1 × 500 µl of 0.6 M aqueous TCA. Subsequently, extracts were centrifuged for 10 min at 16,000 ***g***. The supernatant was transferred to 12 ml tubes, and TCA was removed by extraction with three volumes of diethyl ether. All previous steps were performed at 0–4°C. Remaining traces of diethyl ether were eliminated by placing the samples in a speed vacuum rotor for 10 min.

ATP content in extracts was assayed with a luciferin–luciferase ATP-detection-kit CLSII (Boehringer, Mannheim, Germany) and a Labsystems Luminoskan DS luminometer for 96 multi-well plates. ATP extracts were diluted three times and 10 µl of the diluted extract was added to 100 µl of 0.2 mM tricine buffer (pH 7.6). The CLSII assay mix was injected (50 µl), and photon emission was measured 10 s after injection, for 10 s.

Results

Comparison of ATP visualization and ATP extraction in wild-type seeds

The visualization of ATP can yield data about total amounts of ATP present in a tissue, but may also reveal the tissue-specific distribution. At each imbibition interval shown, 3–4 sections per seed were cut from a total of ten seeds. ATP concentrations in single seeds were calculated by averaging the ATP concentrations as measured in those 3–4 sections. ATP concentrations at each imbibition interval were calculated by averaging ATP concentrations found in single seeds. The average ATP concentrations found in these single seeds varied over a 2.5- to fivefold range between seeds of the same genotype and imbibition intervals (data not shown). To establish whether the determination of ATP concentrations in our seed system was a reliable method, a TCA ATP extraction was performed (Saglio and Pradet, 1980) at the same imbibition intervals with the same seed batch. ATP concentrations obtained with both methods were compared and yielded similar patterns of ATP accumulation during imbibition. ATP could be

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detected at 3 h of imbibition and increased rapidly until 17 h. ATP concentrations did not increase further between 17 and 48 h of imbibition (Fig. 2A, B). At 48 h of imbibition, 50% of the seeds had completed germination (i.e. showed radicle protrusion). Germinated and non-germinated seeds were pooled for the ATP extraction. ATP concentrations in sections of germinated and non-germinated seeds at 48 h did not differ significantly ($P > 0.05$).

ATP distributions were visualized in cryosections of seeds at 3, 6, 17, 24 and 48 h of imbibition (Fig. 3A–E). At 48 h of imbibition, 60% of seeds from this batch had completed germination (i.e. showed radicle protrusion). Cutting cryosections of dry seeds, or seeds at very early stages of imbibition (i.e. prior to 3 h of imbibition), was not successful due to insufficient hydration of the seeds. Both the embryo and endosperm contained ATP during germination. However, the apparent ATP concentration in the embryo was higher compared to the endosperm (average of 1.5 times higher at 24 h of imbibition). Within the embryo the highest concentration of ATP was located in the radicle at all stages of imbibition (Table 1; Figure 3). The ATP concentration in the radicle at the different imbibition intervals was 1.3 to 2.0 ($P < 0.05$) times higher than the ATP concentration in the cotyledons (Table 1).

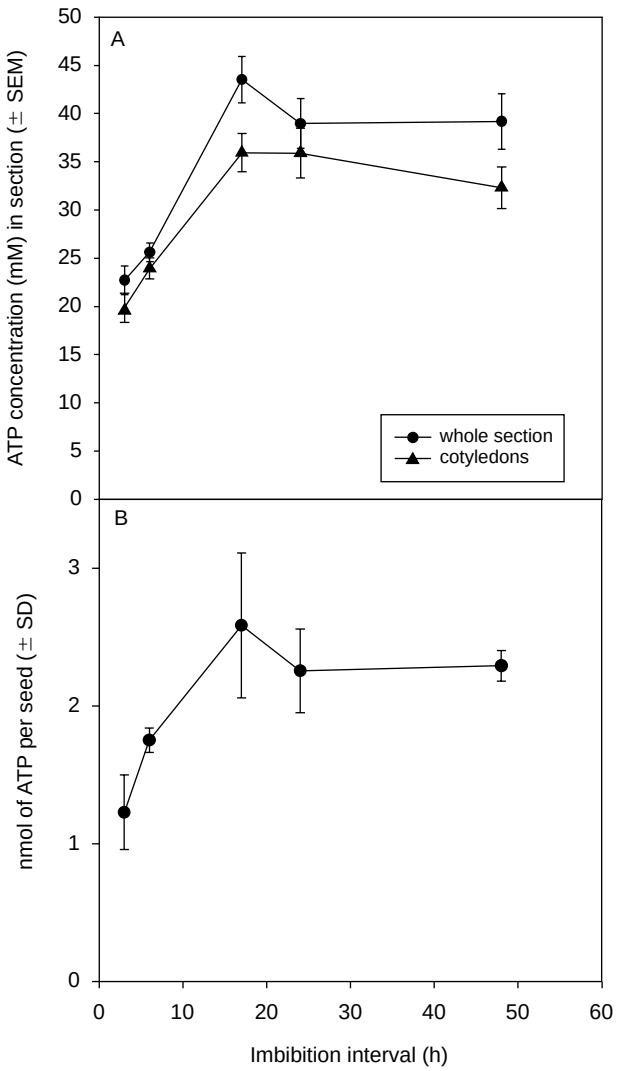

Figure 2. (A) The apparent concentration of ATP during germination of wild-type seeds, as assessed by ATP imaging with luciferase and luciferin. Bars indicate the standard error of the mean. (B) ATP content during germination of wild-type seeds, as assessed by ATP extraction. Bars indicate standard deviations.

ATP distribution in dormant and germinating seeds

ATP imaging of sections of single seeds revealed variation of 2.5- to fivefold in concentration among sections and seeds (results not shown). The ATP concentrations in Table 1 are averages of all sections and seeds per treatment. However, the images shown in Fig. 3 were randomly chosen to depict this variation. Independent of this variation, the ratio of ATP concentrations of radicle and cotyledons was invariably correlated with the physiological state of the seed (dormant versus germinating).

Freshly harvested seed batches may contain proportions of seeds in a state of primary dormancy. Non-dormant, transgenic 35S::luciferase seeds showed luciferase activity during germination, whereas dormant seeds did not (results not shown). This observation was used to distinguish dormant and non-dormant seeds in freshly harvested seed batches. Seeds were imbibed for 48 h in 0.1 mM luciferin and screened for luciferase activity. Cryosections from seeds with primary dormancy were cut and ATP distributions were imaged. As in seeds with secondary dormancy, the ATP concentration in the radicle was comparable to that in the cotyledons (Table 1; Fig. 3H).

Secondary dormancy was induced by far-red light irradiation (De Castro *et al.*, 2001), and ATP was visualized in these transgenic seeds. They displayed an ATP distribution that was clearly different from that of the germinating seeds (Table 1; Fig. 3F). ATP was distributed more evenly in seeds with secondary dormancy than in germinating (wild-type) seeds, resulting in an ATP concentration in the radicle comparable to that in the cotyledons, as in seeds with primary dormancy. A cold treatment was used to break the secondary dormancy, which resulted in 100% germination within 72 h of incubation after transferring the seeds to 25°C. ATP distributions were imaged in cryosections at 24 h after transferring these seeds from 5 to 25°C. The earlier observed pattern of ATP distribution in germinating seeds, with the highest concentration located in the radicle, was

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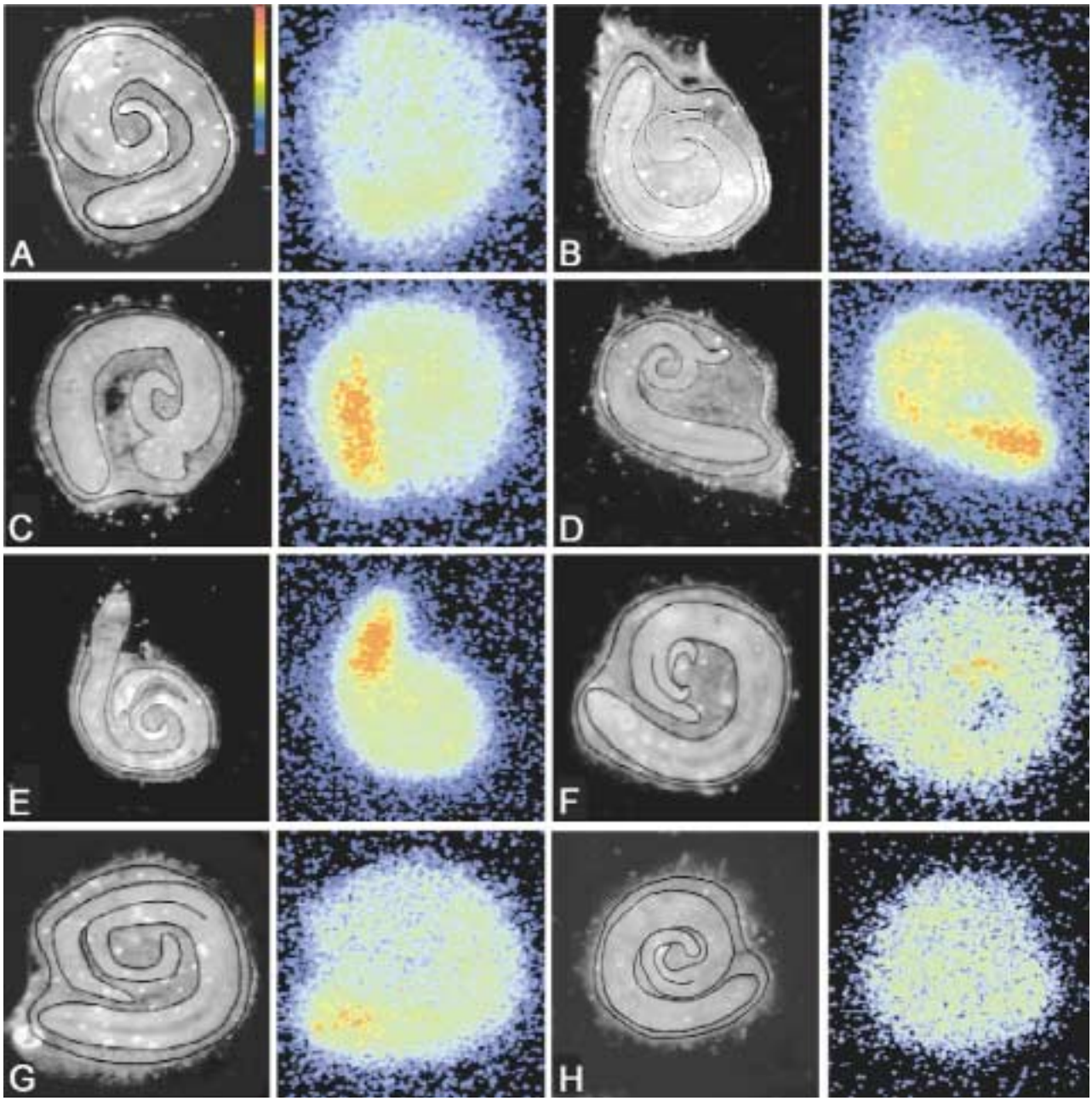

Figure 3. Images of ATP distributions in cryosections of tomato seeds. Wild-type seeds at 3 h (A), 6 h (B), 17 h (C), 24 h (D) and 48 h germinated (E). Secondary dormant wild-type seed after 5 d of incubation (F). Wild-type seed after secondary dormancy induction and cold treatment for 3 d and 24 h at 25°C (G). 35S::luciferase seed in state of primary dormancy at 48 h of imbibition (H). Images were chosen randomly to indicate the variation in ATP concentration among sections. Images on the left are bright-field exposures to indicate the orientation of the seed and embryo depicted on the right pseudo-colour scale. Scaling colour bar in (A) indicates low (blue) to high (red) ATP concentrations. Magnification approximately ×10.

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Table 1. Apparent ATP concentrations in the radicle and cotyledons of wild-type tomato seeds and corresponding standard errors of mean (SEM); ratio of ATP concentration in the radicle and cotyledons; and the statistical significance between the radicle and cotyledons

	ATP concentration (mM)					
	Radicle	SEM	Cotyledons	SEM	Ratio	Significance
3 h Germination	28.0	2.1	19.9	1.5	1.4	$P < 0.05$
6 h Germination	31.6	2.3	23.9	1.1	1.3	$P < 0.05$
17 h Germination	73.5	5.4	35.9	2.0	2.0	$P < 0.05$
24 h Germination	52.3	4.0	35.9	2.6	1.5	$P < 0.05$
48 h Germination	50.4	5.2	32.3	2.1	1.6	$P < 0.05$
Primary dormant	45.6	3.2	42.3	1.3	1.1	$P > 0.05$
Secondary dormant	63.9	5.3	60.3	4.6	1.1	$P > 0.05$
Cold treated, 24 h	112.7	10.3	69.3	6.3	1.6	$P < 0.05$

restored (Table 1, Fig. 3G). The ATP concentration in the radicle was 1.6 times higher compared to the average ATP concentration in the cotyledons ($P < 0.05$).

Discussion

ATP visualization and ATP extraction yield comparable results when studying total ATP content during germination

In the present study, we demonstrated the usefulness of the firefly luciferase–luciferin system to image the ATP distribution in tomato seeds. This technique was modified after an earlier study of ATP imaging in tumour spheroids (Walenta *et al*., 1990). ATP concentrations varied over a 2.5- to fivefold range between single seeds of the same genotype and treatment. Individual seed to seed variation has been demonstrated previously by Still and Bradford (1997), who observed a variation of 4–5 orders of magnitude in endo-β-mannanase activity in single endosperm caps of tomato seeds. Clearly, seed to seed variation is likely to be found for a wide range of metabolite levels and enzyme activities.

The pattern of ATP accumulation during germination of wild-type seeds, as assessed through imaging, was comparable to the accumulation of ATP as determined by the classical method of ATP extraction. The apparent ATP concentrations as assessed by the imaging method seemed to be inconsistent with the total amounts of ATP extracted with the conventional method. However, when calculating the amount of ATP per seed from the images, values are obtained that are of the same order of magnitude as the amounts of ATP extracted, considering that only a limited volume of the whole seed, namely the cytoplasm, contains ATP. Moreover, the ATP-imaging method may be more reliable because the ATP pool at any time-point during imbibition is instantly frozen and fixed, whereas the extraction method may yield underestimates of the ATP pool because of a recovery below 100% and the high turnover of ATP. Nevertheless, since the efficiencies of both methods are not known, ATP concentrations must be considered as apparent.

The accumulated ATP content in seeds was not correlated with germination. For example, the seeds with secondary dormancy displayed an even higher concentration of ATP compared to the germinating wild type. The wild-type seed batch was harvested 7 years before the transgenic seed batches. Comparing dormant seeds with seeds from the same batch that received a cold-treatment to break dormancy, showed higher concentrations in the latter. Thus, absolute ATP levels do not necessarily correlate with germination. ATP concentrations may also vary with genotype and seed lot.

Germination of tomato seeds correlates with ATP distribution but not with the total extractable ATP pool

Earlier studies attempting to show a correlation between seed ATP content and dormancy or germination have yielded conflicting results (i.e. a correlation was not always found). These studies dealt with a large number of plant species, in which different biological processes, such as dormancy, ageing and seed vigour, were examined (Ching, 1973; Lunn and Madsen, 1981; Jain *et al*., 1983; Siegenthaler and Douet-Orhant, 1994). Several causes for contradictory results have been suggested (Mazor *et al*., 1984; Perl, 1986). A likely cause is the high rate of ATP turnover in the cells. Based upon respiration data of Dahal *et al*. (1996), we estimate that a single tomato seed, at 24 h of imbibition at 25°C, produces approximately 7 nmoles of ATP per min. Combined with our data at 24 h of imbibition at 25°C, which showed an extractable pool of ATP of 2.25 nmoles per seed, this suggests that the whole ATP pool within a

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seed is turned over within minutes. This implies that accumulated ATP concentrations and changes therein are negligible compared to actual amounts of ATP synthesized and turned over. Thus, the ATP pool size does not necessarily reflect metabolic rates in a tissue, since the ATP pool is a result of the balance between processes of synthesis and utilization (Perl, 1986).

The present results do suggest that the distribution of ATP within a seed is correlated with germination, rather than the ATP concentration in whole seeds *per se*. Germinating wild-type seeds showed a typical distribution of ATP, with the highest amounts localized in the radicle (ATP concentration in the radicle was 1.4–2.0 times higher than in the cotyledons). Dormant seeds exhibited a more even distribution of ATP, with equal concentrations in radicle and cotyledons. Germination processes that utilize energy in the form of ATP, such as DNA replication (Liu *et al*., 1994; De Castro *et al*., 2000), cell division, microtubule assembly (De Castro *et al*., 2000) and synthesis of endo-β-mannanase (Toorop *et al*., 1996), occur in the radicle during germination of wild-type seeds, but are absent in dormant seeds. In this respect, a higher ATP content in the radicle of germinating seeds might reflect higher metabolic activity, as opposed to the absence of the above processes in dormant seeds.

Conclusion

From the present study, it is clear that the distribution of ATP within tomato seeds, rather than the total ATP concentration within whole seeds, is related to germination. Data presented here could not have been obtained through extraction of ATP from whole seeds; the higher ATP concentration in the radicle is not necessarily reflected in a larger extractable pool of total ATP. Clearly, the study of seed physiology can benefit from techniques that provide detailed information about the *in vivo* spatial distribution of metabolites within seeds.

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