ON THE SEQUENTIAL DETERMINATION OF α -AMYLASE TRANSPORT AND CELL WALL BREAKDOWN IN GERMINATING SEEDS OF HORDEUM VULGARE

by

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By means of immunohistochemical α -amylase determination techniques and Calcofluor white M2R New staining of residual endosperm cell walls it was possible to observe transport of α -amylase and breakdown of cell wall material in germinating seeds of *Hordeum vulgare* cv. Nordal.

When barley seeds of which the distal tip had been removed, were treated with gibberellic acid, the importance of the distal aleurone cells in the production of both α -amylase and cell wall breakdown hydrolases was clearly seen.

1. INTRODUCTION

The capability to observe the transport pattern of de novo synthesised α -amylase during germination and early seeding growth of barley has recently been attained by immunohistochemical means (GIBBONS, 4, 5). The method involved use of a monospecific antibody preparation obtained by immunisation of rabbits with pure α -II (germination specific) α -amylase isoenzyme. By following α -amylase synthesis and transport in germinating seeds of Nordal barley, it could be shown that the scutellum appeared to play a major role in α -amylase dissemination in the

first 3 days of germination, while in contrast to currently accepted theories, the aleurone layer was first a major α -amylase contributor only from the 4th day – a stage at which the enzyme had filled over a third of the endosperm (4).

The successful use of Calcofluor white M2R New in the investigation of plant cell walls of oats and barley (FULCHER et al., 1979, 3) prompted use of this stain to follow cell wall breakdown during germination. Calcofluor white M2R New, a commercial optical brightener, has been shown to react specifically with hexopyranose polymers with β -configuration

(MAEDA and ISHIDA, 8), and intense fluorescence has been reported for barley endosperm cell walls (WOOD and FULCHER, 10). Calcofluor has also been successfully employed in structural studies of vegetative plant organs (HUGHES and MCCULLY, 6). The present communication describes a method for examining cryotome sections of germinating barley seeds, first immunohistochemically for α -amylase transport and secondly for cell wall breakdown.

2. MATERIALS AND METHODS

2.1. Chemicals

Calcofluor white M2R New (di-sodium 4,4'-bis (4-anilinobis-2-hydroxy-ethyl amino-S-triazin-2-ylamino)-2,2'-stilbene-disulfonate) was obtained from Cyanamid, U.S.A., via Struers, Denmark. Gurr Fast Green FCF was obtained from Searle, High Wycombe, England. 96 % ethanol was obtained from De Danske Spritfabrikker, Denmark and fluorescence free glycerol was from Merck, Germany.

Gibberellic acid (grade III-min. 90 % Gibberellin A₃) was obtained from Sigma, U.S.A.

All other chemicals were analytical reagent grade.

2.2. Plant Material

Seeds of *Hordeum vulgare* L. (cv. Nordal) were obtained from Dr. J. LARSEN, Carlsberg Plant Breeding, Denmark. Seeds were pressed into sterile sand moistened with sterile distilled water and germinated for the appropriate time at 15 °C (98 % relative humidity). In the experiments where gibberellic acid was employed the sand was moistened with the appropriate gibberellic acid solution.

2.3. Tissue Preparation

Seeds were removed from the germinating dishes and fixed in 10 % lysine-periodate-paraformaldehyde fixative as described previously (5). Following washing and freezing of the seeds in liquid nitrogen, 15 μ sections were made on a Reichert Jung Frigocut 2700 Cryotome (Jung-Germany). Sections were transferred to moist gelatine-coated slides as described previously (4).

2.4. Immunohistochemical techniques

Immunohistochemical localisation of α -amylase was performed as described by GIBBONS (5).

2.5. Cell-wall measurement

Following microphotography of the anti- α -amylase treated sections, the slides were immersed in phosphate buffered saline (PBS – 0.1 M-Na phosphate buffer, pH 7.4 containing 0.15 M-NaCl) until the coverslips fell off the sections. Sections were further washed for 30 min. in fresh PBS. The slides were then tapped gently to remove excess PBS and treated with 0.01 w/v % aqueous Calcofluor white M2R New for 1 min. Following two washes in 70 % (v/v) aqueous ethanol of 1 min. duration, the sections were counterstained in 0.1 % w/v aqueous Fast Green FCF for 1 min. Excess Fast Green FCF was removed by three 2 sec. washes in 70 % (v/v) aqueous ethanol and allowed to drip dry on filter paper. The sections were finally mounted in glycerol adjusted to pH 7.4 with Na-phosphate buffer.

2.6. Fluorescence microscopy

A Reichert Univar microscope (Reichert, Austria), fitted with a 200 w DC mercury lamp (Illumination Industries Inc.) and epi-illumination was employed throughout. Excitation filters, dichroic mirrors and emission filters were custom made by Optisk Laboratorium (Lundtofte, Denmark) and had the following characteristics: Immunofluorescence – Band Pass BP 455–490 nm exciter, Beam splitter BS 510 nm dichroic mirror, long wave pass LWP 515 nm emission filter; Calcofluor fluorescence – short wave pass SWP 400 nm exciter, Beam Splitter BS 400 nm dichroic mirror, KV 418 nm emission filter. All photomicrographs were taken on Kodak Ektachrome 400 film (Kodak, U.S.A.) using a Reichert Trimatic automatic camera unit (Reichert, Austria).

3. RESULTS AND DISCUSSION

3.1. Normal Germination

Sequential determination of α -amylase transport and cell wall breakdown was carried out using seeds of Nordal barley germinated on

moist sand for periods of up to 7 days. The results obtained by examining longitudinal sections (Figure 1) were similar to those seen previously for α -amylase transport alone (4).

Up to the third day of germination the scutellum played a major role in the mediation of both α -amylase dissemination and the factors effecting endosperm cell wall breakdown. The pattern of cell wall breakdown was the same as that of α -amylase with some indication that the cell wall breakdown slightly preceded the arrival of α -amylase in any given cell. As the major component of barley endosperm cell walls is β -glucan (see review by McLEOD, 7) this would indicate that β -glucanases and other glucan breakdown enzymes must precede α -amylase in the endosperm. This, however, can only be absolutely demonstrated or revoked by the use of immunoelectronmicroscopic studies using antibodies to both α -amylase and β -glucanase.

The active function of the aleurone layer in both α -amylase synthesis and transport, as well as mediating cell wall breakdown is clearly seen in the 124 h and 144 h sections (Figure 1 A 5 + 6 and 1 B 5 + 6).

In the 30 h, 52 h and 76 h sections both α -amylase transport and cell wall breakdown appear to proceed most rapidly in the ventral regions of the seed. This was previously taken as an indication of either a higher concentration of α -amylase in this region, or facilitated transport of enzyme in the ventral endosperm (4).

In order to determine the possible influence of steric factors on the observed hydrolase transport patterns, it was judged necessary to examine serial transverse sections of germinating barley seeds at various stages of germination. Cell wall breakdown analysis was carried out on sections of 30 h, 52 h and 124 h germinated seeds, and immunohistochemical α -amylase determination was performed on sections of the 52 h seeds (Figure 2). There was a marked difference in the pattern of cell wall breakdown between the 30 h + 52 h and the 124 h material. In the former, as successive transverse sections reveal an increasing area of intact cell walls (Figures 2 A, 2, 3 & 4 and 2 B, 3, 4, & 5), cell wall breakdown is seen to occur in the area closest to the scutellum and spreading over the dorsal side of the grain, as well as in a circular region surrounding the

Figure 1. Transport of α -amylase and cell wall breakdown in germinating barley seeds as seen in longitudinal sections.

Column A – Immunohistochemical localisation of α -amylase as described under Methods (2.4). Column B – Calcofluor white M2R New staining of the residual cell walls of the same sections as shown in column A (Methods as described under 2.5). Row 1 – ungerminated; Row 2 – 30 h germinated; Row 3 – 52 h germinated; Row 4 – 76 h germinated; Row 5 – 124 h germinated; Row 6 – 144 h germinated; Row 7 – 173 h germinated. Magnification – $10\times$.

Figure 2. Breakdown of cell wall material in 30 h, 52 h and 124 h germinated barley seeds and α -amylase transport in 52 h germinated seeds as seen in serial transverse sections.

Sections were stained for residual cell wall material as described under Methods (2.5) and for α -amylase as described under Methods (2.4). In all photomicrographs the magnification is $16\times$. Column A – 30 h germinated – transverse sections of the seed taken at: Row 1 – 1806 μ , Row 2 – 2380 μ , Row 3 – 2842 μ , Row 4 – 3290 μ , Row 5 – 3710 μ , Row 6 – 4550 μ , Row 7 – 5558 μ , Row 8 – 6258 μ and Row 9 – 7154 μ from the embryo end of the seed. Total seed length – 8134 μ . Column B – 52 h germinated – transverse sections of the seed taken at: Row 1 – 1918 μ , Row 2 – 2478 μ , Row 3 – 3066 μ , Row 4 – 3612 μ , Row 5 – 4172 μ , Row 6 – 4886 μ , Row 7 – 5446 μ , Row 8 – 6580 μ and Row 9 – 7140 μ from the embryo end of the seed. Total seed length – 8442 μ . Column C – 52 h germinated – transverse sections of the seed (anti- α -amylase treated) taken at: Row 1 – 1638 μ , Row 2 – 2198 μ , Row 3 – 2618 μ , Row 4 – 3080 μ , Row 5 – 3598 μ , Row 6 – 5012 μ , Row 7 – 5586 μ , Row 8 – 6706 μ and Row 9 – 7266 μ from the embryo end of the seed. Total seed length – 8442 μ . Column D – 124 h germinated – transverse sections of the seed taken at: Row 1 – 1988 μ , Row 2 – 2408 μ , Row 3 – 2842 μ , Row 4 – 3164 μ , Row 5 – 3304 μ , Row 6 – 4942 μ , Row 7 – 5740 μ , Row 8 – 6328 μ and Row 9 – 6902 μ from the embryo end of the seed. Total seed length – 8330 μ .

A

B

1

2

3

4

5

6

7

A

B

C

D

1

2

3

4

5

6

7

8

9

central nucellar sheaf cells (see arrow in Figures 2 A, 4 and 2 B, 5). This result indicates strongly that transport of cell wall breakdown enzymes and α -amylase (Figure 2 C, 4 & 5) is facilitated in the cells surrounding the nucellar sheaf. The cells within the nucellar sheaf appear resistant to breakdown (cf. BRIGGS, 1 & 2). In the 124 h germinated seeds a difference in the residual cell wall pattern is seen (Figures 2 D, 7 & 8). Here cell wall breakdown occurs all the way around the central endosperm, indicating aleurone mediated hydrolase synthesis whilst the region directly around the nucellar sheaf cells has no longer the ring shaped area of breakdown (Figure 2 D, 9) seen in the 30 h and 52 h seeds. On the basis of these observations it is tempting to suspect a possible role of area surrounding the nucellar sheaf cells as a transport pathway for scutellar disseminated enzymes during the first 3 days of germination. That the nucellar cells themselves are directly involved in this transport appears less likely, as only small amounts of a α -amylase are observed in them when sections are treated with anti- α -amylase (Figures 2 C 1-5).

The highly asymmetric pattern of α -amylase transport and cell wall breakdown observed in Figure 2 provides therefore some explanation to the patterns previously obtained from the vertical longitudinal sections seen in Figure 1. Slight differences in the cutting angle of a vertical longitudinal section can thus have an undue influence on the findings regarding the shape of the leading edge of the modified area of the grain.

3.2. Gibberellic Acid Treatment

A possible criticism of the immunohistochemical technique of measuring α -amylase transport during germination, is that the isolated antigen - α II- α -amylase is a scutellum-specific

α -amylase and therefore has no relation to the α -amylase produced by aleurone cells during germination. If this were the case, it would be obvious that results obtained using an antibody to this antigen would only show transport of α -amylase out of the scutellum. In order to rule out this possibility a series of germinations were performed in the presence and absence of gibberellic acid using both whole seeds and seeds with a small region of the distal tip cut off. In the latter seeds, treatment with gibberellic acid should initiate α -amylase synthesis in the distal aleurone cells as described by PALMER (9) for »abraded« seeds. If the anti- α II- α -amylase reacted with this aleurone synthesised α -amylase then the criticism that the antibody may only be reacting with a scutellar mediated enzyme, could not be valid. The experimental data (Figures 3 C, 4 & 5, 3 E, 4 & 5) clearly show that the anti- α II- α -amylase reacts with aleurone synthesised α -amylase.

Cell wall breakdown was studied concurrently with α -amylase transport (Figures 3 B, 3 D & 3 F). In order to simplify the figure, only the data for 2, 3, and 4 days germination are shown.

The patterns of α -amylase transport and cell wall breakdown for the untreated control seeds (Figure 3.1) are comparable to those shown in Figure 1 while treatment of whole seeds with gibberellic acid (Figure 3.2) had no significant effect until after 3 days of germination. Decapitation of the distal end had no apparent effect on either the formation and transport of α -amylase, or the breakdown of endosperm cell walls (Figure 3.3). When distally decapitated seeds were treated with a low concentration of gibberellic acid ($1\mu\text{M}$ -Figure 3.4) a marked aleurone-mediated synthesis of α -amylase and breakdown of endosperm cell walls in the distal region was observed after 3 days of germination (Figure 3.4

Figure 3. The pattern of α -amylase transport and cell wall breakdown in seeds treated with gibberellic acid.

α -amylase localisation and cell wall measurement techniques were as described under Methods (2.4 & 2.5 respectively). Column A - 2 day germinated - α -amylase. Column B - 2 day germinated - cell wall breakdown; Column C - 3 day germinated - α -amylase; Column D - 3 day germinated - cell wall breakdown; Column E - 4 day germinated - α -amylase; Column F - 4 day germinated - cell wall breakdown.

Row 1 - Control seeds. Row 2 - Gibberellic acid treated ($10\mu\text{M}$). Row 3 - Seeds with the distal tip removed (distal decapitation). Row 4 - Distally decapitated seeds treated with $1\mu\text{M}$ gibberellic acid. Row 5 - Distally decapitated seeds treated with 1mM gibberellic acid. Magnification = $10\times$.

A

B

C

D

E

F

1

2

3

4

5

C + D). On the following day only a small segment in the central area of the endosperm was unmodified. It is interesting to note that after two days of germination only trace amounts of α -amylase can be detected in the distal aleurone cells of the distally decapitated seeds while the scutellar mediated α -amylase has moved out into the endosperm (Figure 3.4 A). A similar result is seen in the case of cell wall breakdown (Figure 3.4 B). At high concentrations of gibberellic acid (1mM-Figure 3.5) the first significant effects of the hormone are again seen after 3 days. On the fourth day, however, α -amylase has filled the entire endosperm and cell-wall breakdown is complete.

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