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Adenylate Energy Pool and Energy Charge in Maturing Rape Seeds[1]

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ABSTRACT

A study of energy state and chemical composition of pod walls and seeds of maturing rape (*Brassica napus* L.) was conducted on two varieties, Victor and Gorczanski. Total adenosine phosphates, ATP, and adenylate energy charge increased with increasing cell number and cellular synthesis during the early stages, remained high at maximum dry weight accumulation and maximum substrate influx time, and decreased with ripening. A temporal control of energy supply and ATP concentration is evident in developing tissues with determined functions; whereas the association of a high energy charge and active cellular biosynthesis occurs only in tissues with a stabilized cell number.

Maturing seeds require a tremendous supply of ATP for the biosynthesis of various seed components, for the biochemical work, such as the loading and transport of phloem sap and minerals and the intracellular cytoplasmic streaming, and particularly for the accumulation of RNAs with the increasing number of cells and cellular activities. It is of interest to measure this energy supply during seed development and maturation and to discern whether or not a regulatory role of the adenylate energy charge is imposed on these processes.

MATERIALS AND METHODS

Materials. Two varieties, Victor and Gorczanski, of rape (*Brassica napus* L.) were planted at a rate of 1.2 kg/ha in a 10-cm row spacing at Corvallis, Oregon, on September 28, 1972. The soil was a silty clay loam with a pH of 5.0 to 5.4. A preplant fertilizer application of 400 kg of N/ha and an additional 100 kg of N/ha on March 6, 1973 were applied. Weed control was effected by preplant incorporation of 4 kg/ha of Eptam (*S*-ethyl dipropylthiocarbamate) and 3.4 kg/ha of Treflan (α,α,α-trifluoro-2,6-dinitro-N'N-dipropyl-*p*-toluidine). No irrigation was applied because the natural rainfall was enough to provide the water needs of the plants. On May 1, 1973, most plants had 50% of the florets at anthesis stage. A representative group of inflorescences with a stalk length of 1.5 to 1.75 m above the soil surface was tagged for both varieties on May 3, 1973. Samples were taken on May 8, 14, 22, 29, June 4, 13, and July 15. On the last sampling date, most pods of Gorczanski were yellow, but some were greenish yellow in Victor.

Sample collection. Two representative racemes were selected for each sampling date for each variety. The pods on one side of the raceme were cut off and immediately dropped into liquid nitrogen for chemical analyses in the laboratory. Because of the rapid change in the contents of adenosine phosphates in tissues, liquid N_2 was used to maintain their *in situ* content during transport. The other half of the pods were kept in polyethylene bags in crushed ice for the determination of weight, size, and water content. The time of sample collection was consistently maintained around 9:00 AM to minimize diurnal variation.

Extraction of Adenosine Phosphates (AP). Pods in liquid N_2 were poured into a supercooled aluminum pan, packed in Dry Ice. The pods were classified into size classes of 1-cm increments and the number of pods in each class was recorded. Pods smaller than 5 cm in length were extracted whole because the seeds were inseparable from the pod wall. Larger pods were dissected to pod walls and seeds under a frozen condition, then extracted separately. At least two replications were analyzed for each size class and a total of 8 to 20 analyses were conducted per sampling date of each cultivar. About 200 mg of the fresh weight of pod walls or seeds were ground with a piece of Dry Ice and 2 ml of 0.25 N perchloric acid ($HClO_4$) in a supercooled mortar with pestle (20). In previous studies, the boiling water extraction provides higher recovery of adenylates and more reproducible results than any of the other extraction methods (7). The advantage of a boiling temperature in stopping enzyme activity and extracting soluble compounds cannot be achieved on materials of large quantity and at liquid N_2 temperature. Therefore, perchloric acid was chosen as the extractant. An additional 3 ml of $HClO_4$ were added into the slurry and the mixture was transferred into a polyethylene tube. Another 3 ml of $HClO_4$ were used to rinse the mortar and pestle and the rinse was added into the polyethylene tube. The tube was centrifuged at 10,000*g* for 5 min and the supernatant was collected. The residue was reextracted with 2 ml of $HClO_4$ and the supernatants were combined. The two $HClO_4$ extractions removed about 95% of the total nucleotides with a distribution of 85 to 90% in the first extraction and 5 to 10% in the second extraction. The supernatant was neutralized with KOH and $KClO_4$ was removed by centrifugation. The neutralized extract was made to a volume of 15 ml. All procedures were conducted at 0 to 3 C.

Assay of ATP, ADP, and AMP. An aliquot of the neutralized extract was diluted to 10X with a buffer containing 0.025 M HEPES and Mg^{2+} acetate, pH 7.5. This diluted ex-

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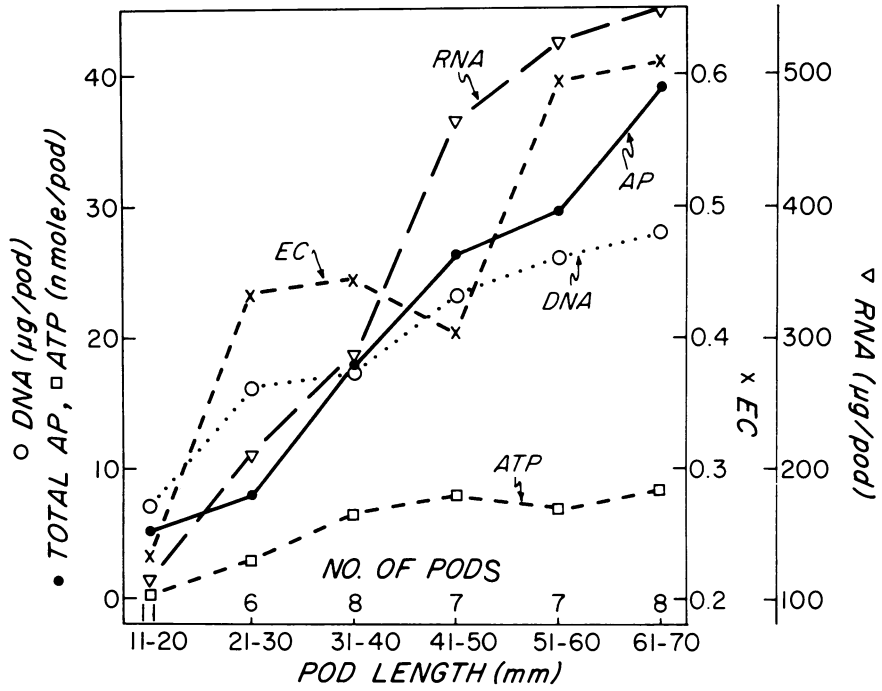

FIG. 1. Contents of ATP, total AP, DNA, and RNA and EC in whole pods of different lengths collected 8 days after anthesis from two longitudinal half-racemes of the variety Victor.

tract can be kept at 0 to 4 C without deterioration for at least 1 day. ATP, ADP, and AMP were assayed by the luciferin-luciferase system (7) with one exception, that an internal standard was added to correct quenching (24). Generally, the quenching is less than 20%.

Analyses of Chemical Composition. Soluble sugars, free amino acids, and inorganic phosphate were determined in the neutralized extract by the anthrone, ninhydrin, and Fiske-SubbaRow method, respectively (6). DNA and RNA were assayed by the diphenylamine and orcinol procedures in the $HClO_4$-extracted residue (6). Total protein was estimated in dried materials by the micro-Kjeldahl method with a *N*-protein factor of 6.25. Lipids were extracted with chloroform-methanol (2:1, v/v) from frozen materials and estimated gravimetrically. Insoluble nonstructural carbohydrate was estimated by the anthrone method in lipid-free residue hydrolyzed with 0.2 N H_2SO_4 (23).

Fresh Weight, Dry Weight, and Water Content. Pods collected in polyethylene bags were placed on a moist paper towel, measured, and classified to size groups. Seeds were separated from the pod wall and placed in weighing bottles, their fresh weight was recorded, and dry weight was obtained after drying at 100 C for 24 hr. The water content was calculated as the per cent of fresh weight.

RESULTS AND DISCUSSION

Because of the indeterminate flowering habit of rape, the physiological stages of individual pods on a raceme varied a great deal, especially in the early stages of development. The data in Figure 1 indicate such variation. At about 8 days after anthesis, the seeds were mostly inseparable from the pod wall, so the whole pod was analyzed. It is shown clearly that the contents of DNA, RNA, total AP, ATP, and the EC[2](EC = (ATP) + ½(ADP)/(ATP) + (ADP) + (AMP)) of the whole pod increased with the pod length. One coordinated sharp increase of RNA and a decrease of energy charge in the pod length class 41 to 50 mm is of interest because it might indicate that the accumulation of RNA is at the expense of ATP. In the later stages of development, most pods on a raceme were of larger and uniform sizes. However, in order to present the over-all data for the whole raceme, a weighted average per pod walls or per seeds from one pod was calculated for each sampling date. The weighted average was calculated based on the number of pods in different classes.

Both varieties increased their fresh weight with increasing time after anthesis to about 40 days, then decreased (Fig. 2A). Their dry weight accumulated continuously with the pod wall reaching a plateau at about 35 days and the seeds at 50 days after anthesis (Fig. 2B). The water content in both tissues decreased with increasing time after anthesis with the pod wall declining at a slower rate than the seeds (Fig. 2C). These changes are common to maturing pods and seeds of other species (11, 12). The accumulation of lipids (Fig. 2D) followed the same pattern of other varieties of rape seeds, except that the oil contents were somewhat higher in these two varieties (15). All of these figures indicated that the crop developed and ripened normally.

The increase of RNA (Fig. 2E) in the pod tissue coincides with the developmental needs of ribosomal, transfer, and messenger RNAs in synthesizing cellular constituents and particularly amino acids for the accumulation of reserve protein in rape seeds (Fig. 2H). After the developmental needs were met, the content of RNA (Fig. 2E), DNA (Fig. 2F), sugars (Fig. 2G), protein (Fig. 2H), and amino acids (Fig. 2I) in the pod wall all declined rapidly. The RNA in the seeds of one pod increased continuously up to 35 days old (Fig. 2E), then remained constant to full maturity. Even though a quantitative difference in RNA was not observed in rape seeds with maturity during later stages, qualitative changes among the three kinds of RNA might exist as in maturing peas (3). The continuous increase of RNA in seeds lagged slightly behind the accumulation of DNA (Fig. 2F) indicating a normal pattern of synthesis in cells of the developing embryo.

Soluble sugars (Fig. 2G) appeared to be the major influxed

[2] Abbreviation: EC: energy charge.

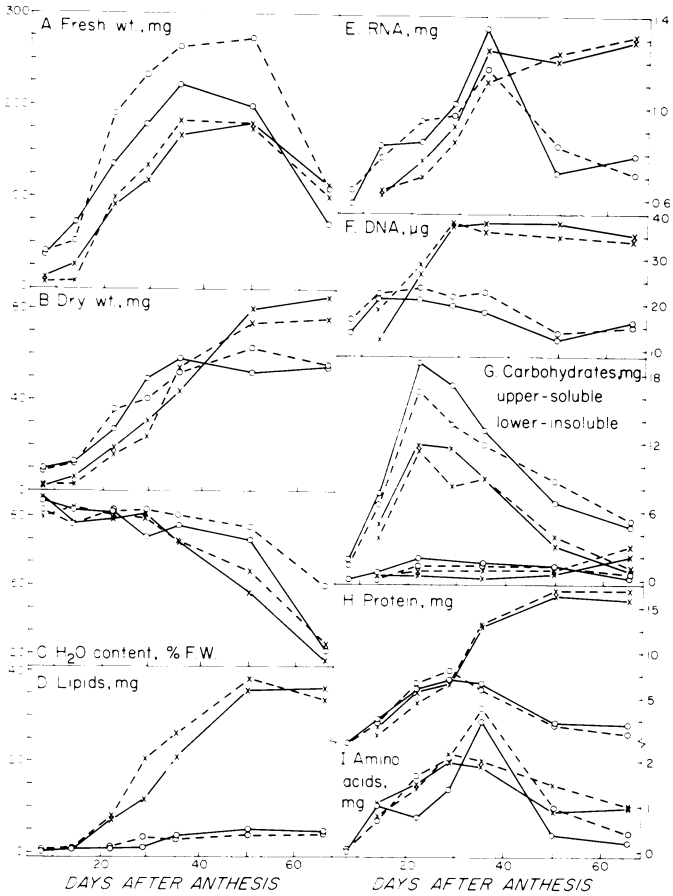

FIG. 2. Changes in fresh weight (A), dry weight (B), and contents of water (C), lipids (D), RNA (E), DNA (F), carbohydrates (G), protein (H), and amino acids (I) of one pod wall (O–O–O) and of seeds in one pod (X–X–X) during maturation of varieties (Gorczanski (——) and Victor (····).

substrate for the synthesis of various seed constituents comprising about 60% of the dry weight of seeds or pods at 14 and 21 days after anthesis (Fig. 2, B and G). The green pod wall and green seeds also produce soluble sugars from photosynthates. The precise proportion contributed by leaves and the pod wall was impossible to ascertain by this study. The fact that after the peak of soluble sugars was reached in 22 days, a rapid increase of RNA, protein, and amino acids was observed in the pod wall, and then in seeds, indicated a sequential transfer as in other species (12, 25). Insoluble but nonstructural carbohydrates were low in quantity in pod walls and seeds during maturation. A small accumulation, however, was noted in the mature seeds (Fig. 2G). This observation is consistent with the composition of other varieties (1).

In the mature seeds, the following chemical composition was observed on a dry weight basis. DNA, 0.04%; RNA, 2%; sugars, 1.4%; insoluble nonstructural carbohydrates, 4.5%; free amino acids, 1.3%; protein, 20%; and lipids, 42%. The difference between the two varieties was very small on the limited samples studied.

The total potential energy pool as measured by total adenosine phosphates in the tissue increased with development both in seeds and pod walls with seeds having a larger peak total content of about 55 nmoles/seeds in one pod and about 37 nmoles/1 pod wall (Fig. 3A). Values for both the pod walls and seeds reached a peak about 28 days after anthesis for both varieties. The AP pool was maintained at a peak state for 1 week and then decreased after 35 days of development in both tissues with a more rapid decline in the pod walls than in seeds. This pattern of rise and fall is correlated with the cellular activities of the tissues as shown by the accumulation rate of various seed components. A similar rise and fall in adenosine nucleotides was observed in developing wheat grain (13).

The cellular activities of developing fruits involve mainly the synthesis of new cell components at the early stages. At the later stages, photosynthesis and the transport of photosynthates and phloem sap are the major functions of the pod walls and the accumulation of reserve compounds in seeds. The increase of cell numbers as indicated by DNA content (Fig. 2F) occurred mainly in the first 14 days in the pod wall and about the first 30 days in seeds. During these periods, the ATP pool (Fig. 3, B and C), the AP pool (Fig. 3A), and the EC (Fig. 3D) all rose rapidly, indicating the coordinated effort of energy metabolism with the increase of cell number in both pod walls and seeds. The increased pools of ATP and AP and the high energy charge in the tissues, however, remained steady, even after the attainment of the maximum cell number to accommodate the needs in the biosynthesis of RNA (Fig. 2E), DNA (Fig. 2F), lipids (Fig. 2D), and protein (Fig. 2H) (3, 17), and in transporting sugars (Fig. 2G), inorganic phosphate (Fig. 3F) (14, 22), and amino acids (Fig. 2I) (16). After the synthetic machineries were fully furnished in the tissues, around 35 days after anthesis, the pool of total AP and ATP began to shrink with a concomitant reduction of the energy charge. The concentration of ATP on a fresh weight basis (Fig. 3E), however, was actually increased in seeds 50 days after anthesis. This increase in concentration probably sustains the energy needs of lipid (Fig. 2D) and protein (Fig. 2H) synthesis (15) in the cytosol of embryonic cells at that late stage

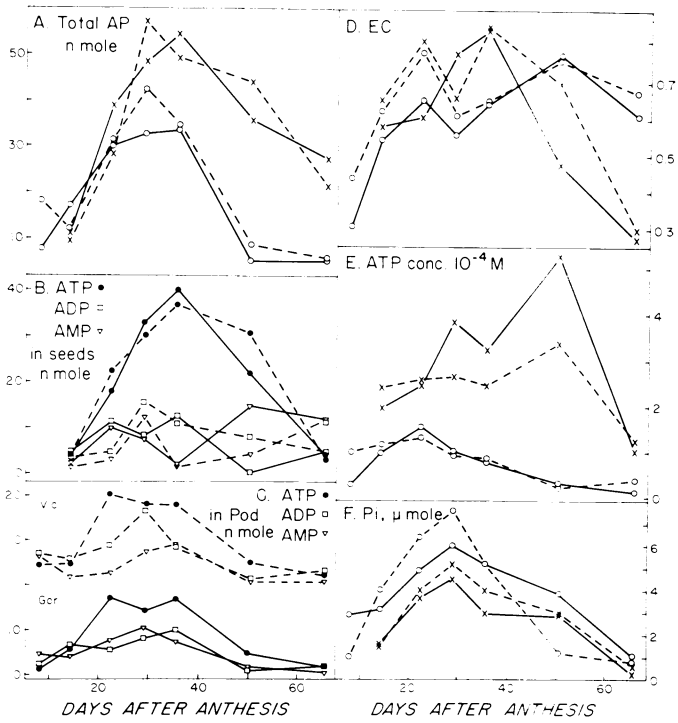

FIG. 3. Changes in total adenosine phosphates (A), ATP, ADP, and AMP in seeds of one pod (B), ATP, ADP, and AMP in the pod wall (C), energy charge (D), ATP concentration (E), and inorganic phosphate (F), of one pod wall (O–O–O) and of seeds in one pod (X–X–X) during maturation of varieties Gorczanski (——) and Victor (····).

of maturation. It appears, therefore, that the concentration of ATP in tissue is an important parameter of biosynthesis when cell numbers are stabilized. Further evidence supporting this conclusion is the nondividing gametophytic tissue of germinating ponderosa pine seeds (7). In that tissue, the ATP concentration increased by 2.5-fold during the peak biogenesis of enyzmes, mitochondria, and glyoxysomes.

Atkinson (2) proposed the concept of EC as an overall measure of the energy state of the cell. He stated that the EC modulates the activity of various metabolic sequences related to energy utilization and regeneration. In general, when the EC is greater than 0.5 ATP-utilizing systems increase their activities. At an EC lower than 0.5 in cells, ATP-regenerating sequences become dominant. Multiplying and growing bacteria cells maintain a high EC of around 0.8, senescent cells have a low EC of 0.5, and cells die at EC values below 0.5 (5). Other work indicates that EC regulates protein synthesis and the growth of bacteria is a well established fact (17). In sycamore cell culture (4), the EC was 0.73 at the lag phase, 0.66 at the exponentially growing stage, and 0.81 at the stationary phase. In germinating ponderosa pine embryos and seedlings (7), a situation parallel to that of the sycamore cell culture is shown. During stratification, cell number was stable and the EC was 0.85. During the early stages of germination, all of the tissues were growing in cell number and size and the EC was 0.65 to 0.75. During the later stages of germination, the EC was high again (0.8–0.9). The increase of cell number was very limited at this stage of growth, although the cell size was increasing rapidly. In pea leaves, EC controls the activity of 3-phosphoglyceric acid kinase but not ribulose 5-phosphate kinase (19). In animal materials, a higher EC was observed in cancer cells than the normal ones (9). Fasting and diabetic rats had lower EC in liver tissue than the control (10).

Whether EC controls the growth of maturing fruits is again as difficult a question to answer as its role in germinating seeds (7). Apparently a low EC is prevalent in meristemic tissues such as the developing fruits reported here (Fig. 1, and 3D) and embryos with high mitotic index in germinating seeds (7). Perhaps, when the cell number is stabilized, then the cellular EC could regulate the growth of the tissue as shown by the correlated increase of the EC in seeds and pod walls (Fig. 3D) and the rate of fresh and dry weight accumulation (Fig. 2, A and B). Further support of this speculation can also be found in the literature. The association of a high EC to rapid growth rate and the synthesizing ability of protein, RNA, and lipids in a fast growing variety of wheat seedlings (8) is one example. Another example is the consistent increase of the EC prior to biosynthetic activities in nondividing or mature tissues, such as the gametophyte and the embryo of germinating ponderosa pine seeds during stratification (7) and the embryos of wheat and lettuce during the first few hours of germination (18, 21). During the later two stages of seed maturation, the EC in the pod tissues was 0.61 to 0.76 but the tissues were being degraded and became senescent as evidenced by the reduction of RNA (Fig. 2E), DNA (Fig. 2F) protein (Fig. 2H) and water content (Fig. 2C). When the EC in seeds was reduced to 0.48 in Gorczanski 50 days after anthesis, a peak concentration of 525 μM ATP was manifested to sustain the peak accumulation of lipids and protein. Interestingly, when the EC in seeds of Victor was not greatly reduced in the later stages of maturation, a moderate elevation of ATP concentration was attained simultaneously. Therefore, a control system of growth with multiple switches apparently is installed in different plant tissues to ensure the accomplishment of the development. How these multiple switches are affected and how they interact are questions to be solved.

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