Short Communication

Isolation of Protoplasts and Chloroplasts from Flag Leaves of
Triticum aestivum L. 1

RICHARD C. LEEGOOD AND DAVID A. WALKER
Department of Botany, The University of Sheffield, Sheffield S10 2TN, England

ABSTRACT

Intact protoplasts and chloroplasts have been isolated from mature flag leaves of wheat (Triticum aestivum L.). Both showed high rates of photosynthesis, the best of which equaled those observed in the parent tissue (greater than 150 micromoles O₂ per milligram chlorophyll per hour). The presence of ethylene-diaminetetraacetic acid and an alkaline medium (pH 8.4) were required in the isolation and assay for the achievement of maximum rates of photosynthesis by chloroplasts. Photosynthesis by isolated chloroplasts was inhibited at very low concentrations of external orthophosphate.

A large part of our present knowledge concerning the movement of metabolites between the chloroplast and its cellular environment derives from experiments with intact isolated chloroplasts. It is now widely accepted that fully functional organelles provide a more realistic basis for studies of electron transport, photophosphorylation etc. than those which have been unintentionally stripped of their limiting envelopes during isolation. Although lack of damage cannot be guaranteed at present, envelope integrity can be inversely related to the ability of isolated plastids to support ferricyanide-dependent O₂ evolution and chloroplasts which display a high degree of intactness and an ability to assimilate carbon at rates which match those of the parent tissue are generally regarded as metabolically competent. It has previously proved possible to isolate chloroplasts with these characteristics from only a few species, such as spinach, but not, e.g. from the flag leaf of wheat. This tissue makes a major contribution to the fixed carbon in the developing grain (5) and its photosynthetic behavior is widely studied. The availability of chloroplasts from this source would greatly facilitate research into the metabolism of a crop species of considerable importance. We wish to report an extension of the method of Edwards et al. (4) which has allowed the separation of highly active chloroplasts from the flag leaf of Triticum aestivum L., var. Sappo.

MATERIALS AND METHODS

Wheat Protoplasts. Wheat (T. aestivum L., var. Sappo) was grown in a greenhouse in a mixture of compost and Vermiculite under sunlight and supplemental lighting at a minimum temperature of 23°C. Flag leaves were removed from plants during the 7 days following anthesis and were used immediately for the preparation of protoplasts. Six to 7 g of laminae were used to prepare strips, 0.5 to 1.0 mm wide, which were hand-cut using a razor blade. The strips were incubated for 3 h in the light at 28°C in 40 ml enzyme medium containing 0.5 mM sorbitol, 1 mM CaCl₂, 0.5% (w/v) BSA, 2.5% (w/v) cellulase (Onozuka S3), 0.3% (w/v) pectinase (Macerozyme), and 5 mM Mes (pH 5.5). The medium was removed and the protoplasts were released from the strips by gently chopping them with the blade of a spatula. The tissue was washed three times with 20-m1 aliquots of 0.5 mM sorbitol, 1 mM CaCl₂, 5 mM Mes (pH 6.0). The protoplasts were collected by centrifugation and purified in a sucrose and sorbitol step-gradient as described by Edwards et al. (4), and were stored on ice in a 0.5 M mixture of sorbitol and sucrose, 1 mM CaCl₂, 5 mM Mes (pH 6.0).

Preparation of Chloroplasts. Chloroplasts were prepared from protoplasts as described by Edwards et al. (4) and were pelleted by centrifugation at 250 g for 40 s. The medium used for isolation and resuspension contained 0.33 mM sorbitol, 10 mM NaHCO₃, 10 mM EDTA and 25 mM Tricine (pH 8.4). For the mechanical preparation of chloroplasts, 20 g leaves, cut into 2-cm segments, were homogenized in 150 ml of semifrozen grinding medium for 4 s in a Polytron blender and the homogenate squeezed through two layers of muslin. The resultant brei was filtered through eight layers of muslin plus cotton wool and centrifuged in a swing-out Christ centrifuge from rest to 6,000 rpm to rest in 90 s. The grinding medium contained 0.33 mM sorbitol, 10 mM NaHCO₃, 10 mM EDTA, 5 mM MgCl₂, 0.1% (w/v) BSA, 0.2% (w/v) sodium d-isoascorbate, and 20 mM Hepes (pH 7.6). Chloroplast intactness was determined by ferricyanide-dependent O₂ evolution before and after osmotic shock (7).

Photosynthetic Assays. CO₂-dependent O₂ evolution was followed polarographically at 20°C in a twin Clark-type electrode system (2) purchased from Hansatech Ltd., Hardwick Industrial Estate, King's Lynn, Norfolk. Photosynthesis by isolated proplasts was measured in a medium containing 0.5 mM sorbitol, 10 mM NaHCO₃, 1 mM EDTA, 1 mM CaCl₂, and 50 mM Hepes (pH 7.6).

Materials. Cellulase and Macerozyme were purchased from Yakult Biochemicals Co. Ltd., Nishinomiya, Japan.

RESULTS AND DISCUSSION

Flag Leaf Protoplasts. Although we isolated protoplasts and chloroplasts from material during the 7 days following anthesis, we encountered no difficulties in the isolation of either in an active state at all stages of flag leaf development. One isolation yielded about 1.0 to 1.5 mg Chl as protoplasts. Since the Chl content of the flag leaves was about 3 mg/g fresh weight, this represents a yield of about 5% on a Chl basis, compared with a yield of about 10% when young wheat leaves are used (6).
The isolated protoplasts showed CO₂-dependent O₂ evolution; the average rate measured over 30 experiments was 86.2 μmol O₂ mg⁻¹ Chl h⁻¹ when assayed under optimum conditions in white light. The variation was quite large; the range was 44 to 176 μmol O₂ mg⁻¹ Chl h⁻¹. These rates of photosynthesis are considerably higher than those obtained by Huber and Edwards (6) using leaves from 21-day-old wheat and the best rates are in the same range as those recorded for the parent tissue under similar conditions of temperature and light intensity. Measurement of apparent photosynthesis in flag leaves gave rates of 150 to 200 μmol CO₂ mg⁻¹ Chl h⁻¹ (A. Herold, unpublished results).

Protoplasts stored at 2 C in the dark in 0.5 mM sucrose, 1 mM CaCl₂, and 5 mM Mes (pH 6.0), showed no loss of photosynthetic activity over a period of 48 h. Chloroplasts isolated from protoplasts stored over this time displayed only low rates of photosynthesis.

**Flag Leaf Chloroplasts.** Until relatively recently, wholly mechanical methods have been the only means of preparing functionally intact chloroplasts and these have been applied successfully only to species such as spinach and peas (10). In the last year or so, protoplasts have been used to prepare chloroplasts from wheat (4) and, in particular, sunflower (3), a species in which mechanical procedures had yielded chloroplasts of only very modest performance despite the inclusion of a great many protective agents in the isolation media (1). We have employed a mechanical method to prepare chloroplasts from young (5- to 8-day-old) leaves of wheat. Such chloroplasts are 70 to 80% intact and have rates of CO₂-dependent O₂ evolution which exceed 100 μmol O₂ mg⁻¹ Chl h⁻¹ and approach those rates obtained in chloroplasts isolated from protoplasts from young wheat leaves (4). We attempted to isolate chloroplasts from flag leaves by this method. The preparations contained no intact chloroplasts (as measured by ferricyanide-dependent O₂ evolution) and consequently showed no CO₂-dependent O₂ evolution. Our inability to prepare functional chloroplasts by the mechanical method indicates the value of the preparation of chloroplasts from protoplasts where "difficult" species such as wheat and sunflower are concerned.

Flag leaf protoplasts yielded chloroplast preparations which were greater than 90% intact and which had photosynthetic rates which usually exceeded 100 μmol O₂ mg⁻¹ Chl h⁻¹ (the highest rate recorded was 156 μmol O₂ mg⁻¹ Chl h⁻¹). Like protoplasts, the best rates of photosynthesis approach those observed in the parent tissue. Rates of photosynthesis by isolated chloroplasts were stable for at least 1 h when the chloroplasts were stored on ice.

Like chloroplasts from young wheat leaves, chloroplasts isolated from flag leaves exhibited a strong EDTA requirement for the achievement of maximum rates of photosynthesis (3, 4). They showed a rather stronger requirement for EDTA and a more alkaline and sharper pH optimum for CO₂-dependent O₂ evolution. The extent of this requirement is shown in Figure 1. When chloroplasts were assayed at pH 8.4, EDTA had no effect upon the observed rates of photosynthesis, although a reduction in the length of the lag occurred when it was present at the higher concentration (Fig. 1, a and c). Isolation at pH 7.6 as opposed to pH 8.4, or assay at low (1.7 mM) as opposed to high (11.7 mM) EDTA extended the lags and decreased the rates of photosynthesis. Isolation or assay at pH 8.0 gave rates intermediate between those shown at pH 7.6 and pH 8.4. The striking extension of the lag caused by isolation or assay at pH 7.6 contrasts with the effect in chloroplasts from young wheat, which is much less marked (3, 4). At all the pH values tested, inclusion of EDTA in the isolation medium was essential to obtain any CO₂-dependent O₂ evolution by these chloroplasts.

A particularly striking property of chloroplasts prepared from flag leaves was their susceptibility to inhibition by Pi (Fig. 2). Photosynthesis showed a fairly sharp and constant optimum at 0.2 mM Pi, but above this concentration extensive inhibition occurred. At 1.0 to 1.5 mM Pi, photosynthesis was almost completely inhibited (the lowest Pi concentration at which complete inhibition was observed was 0.5 mM). Although such Pi curves vary considerably with the pretreatment of the tissue and other factors, such susceptibility contrasts with the situation found in spinach chloroplasts in which 10 mM Pi will sometimes fail to inhibit photosynthesis completely and 0.5 mM Pi is often near optimal (8). In chloroplasts from young wheat leaves and peas inhibition is not nearly as severe at low Pi concentrations. For example, a 50% inhibition of photosynthesis occurs at about 0.5 mM Pi in flag-leaf chloroplasts, but 1.0 to 2.0 mM Pi is required for 50% inhibition in chloroplasts from peas (9) or young wheat (4). Although these
differences may not appear very remarkable, they are reproducible and, bearing in mind the fact that movement of triose-P from the chloroplast is linked to Pi uptake with a stoichiometry of unity, they may well be important in a tissue in which the export of assimilated carbon is a prime function.

LITERATURE CITED