Characterization of a Light-Induced Oxygen-Uptake in Tobacco Protoplasts

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Protoplasts prepared from the wild type tobacco *N. tabacum var. John William's Broadleaf* exhibit photosynthetic oxygen-evolution if the suspension medium is supplemented with bicarbonate. In the absence of bicarbonate no steady state oxygen-evolution is observed with such preparations. Instead, an appreciable uptake which is mainly insensitive to DCMU and which persists over hours, and therefore is no induction phenomenon, is seen. Protoplasts of the tobacco aurea mutant *Su/su*, which is a plant with an exceptionally high photorespiration, show an oxygen consumption in the light which is 4 to 5 times higher per protoplast than in the wild type. Again, the uptake is practically insensitive to DCMU which means that the effect is to be associated with photosystem I. This is further substantiated by the fact that protoplasts prepared from yellow leaf sections of the variegated tobacco mutant *NC 95* also show the light induced uptake. As reported earlier, the yellow leaf sections of this mutant exhibit only photosystem I reactions.

The action spectrum for this oxygen-uptake yields the spectrum of chlorophyll. Consequently, this uptake is an inherent property of the chloroplast and has nothing to do with earlier described, light-dependent oxygen consumptions, which were mainly driven by blue light and hence used some yellow pigment as the photoreceptor. No effect or contribution of yellow pigments such as carotenoids is seen since the action spectrum with the yellow tobacco mutants which have an up to 4 fold higher carotenoid/chlorophyll ratio than the wild type is identical to that of the wild type.

One of the major goals of modern plant breeding in the future must be the suppression of photorespiration. In the process of photorespiration, as known from the literature [1, 2], primary products of photosynthetic CO_2-fixation are dissimilated in a seemingly useless manner. As the amount of photorespiratory oxygen-uptake, as measured by $^{18}O_2$ consumption [3], exceeds manyfold the photorespiratory CO_2-evolution measured as $^{14}CO_2$ [2], a simple oxidative decarboxylation cannot or at least not be responsible for the larger part of the phenomenon. It rather appears that what is generally called photorespiration is a mixed phenomenon in which biochemical processes are sensitive to high partial pressures of oxygen. Moreover, these reactions seem to take place in quite different cell compartments. The only meaningful sense of such oxidative reactions could be seen in the disposal of excess reducing power.

As modern plant breeding uses such methods as protoplast fusions [4], cell organ transfer or their mixing [5], we attempted to prepare functioning protoplasts from the tobacco mutants which we have genetically characterized in an earlier report [6]. In the present paper we have tried to characterize these preparations with respect to reactions that could be associated with photorespiration.

Materials and Methods

Tobacco plants were grown in a green-house. The green wild type was the Connecticut cigar Variety *N. tabacum var. John William's Broadleaf.* The aurea mutants *N. tabacum var. Su/su* and the *Su/su var. aurea* and the variegated tobacco mutant from *N. tabacum var. NC 95* have been described earlier [6, 7].

Protoplast preparations were prepared according to the somewhat modified procedure of Binding [8] by digesting at room temperature fine cut tobacco leaves in the light with macerase and cellulase both from Kingki Yakoult, Japan. In order to obtain protoplasts that are capable of doing photosynthesis it is absolutely necessary to wash them at least 4 – 5 times after the incubation with sea water or the final sus-

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a

b

c

pension buffer in order to remove all traces of the enzyme which otherwise cause the destruction of the protoplasts in the functional test. The quality of the preparation is checked under the microscope before use. The final suspension medium was either 0.3 M mannitol or a buffer containing 0.4 M sucrose, 0.05 M Tris or Tricine pH 7.2, 0.01 M NaCl.

Chloroplast preparations were made for comparison purpose. The preparation method used was that of Homann and Schmid [9].

Chlorophyll determinations have been described earlier [7]. Usually 1 ml suspension medium containing protoplasts equivalent to 100 μg chlorophyll for the wild type or 20 μg chlorophyll for the *Su/su* mutant were used.

The oxygen electrode system used has been described in detail recently [10]. 1 ml of the protoplast suspension was put on the electrode. The suspension was allowed to sediment for 10–15 minutes before measurements. No stirring was applied. All measurements were carried out at room temperature.

Light source and illumination were provided by a 6.5 kW xenon lamp equipped with a monochromator (SOPRA). For the measurement of the action spectrum the individual wavelengths were adjusted to the equal quantum flux of 660 μ Einstein $\cdot$ s^{-1} $\cdot$ m^{-2}. The measurements were normalized to the effect observed at 660 nm. The effect at every wavelength was in the linear range of its dependence on the light intensity.

Results

Protoplasts of the wild type tobacco N. tabacum var. John William's Broadleaf. Protoplasts prepared from wild type tobacco *N. tabacum var. John Williams's Broadleaf* exhibit a light induced oxygen-evolution which is DCMU-sensitive provided the protoplasts are incubated with 0.1 M sodium bicarbonate at around pH 7.2 (Fig. 1). The oxygen evolution seems to require a preillumination period of 1 – 2 minutes

Fig. 1. Oxygen gas exchange in a protoplast preparation of wild type tobacco *N. tabacum var. John William's Broadleaf.* Illumination with monochromatic light 660 nm. The 1 ml suspension on the electrode surface contained protoplasts corresponding to 100 μg chlorophyll and 0.1 M bicarbonate. a) Illumination of dark adapted assay; b) the same assay reilluminated after 3 min of darkness; c) as b) but reilluminated after 3 min of darkness.

Fig. 2. Oxygen gas exchange in wild type tobacco protoplasts without bicarbonate addition otherwise conditions as in Fig. 1. Chlorophyll content on the electrode 123 µg/ml.
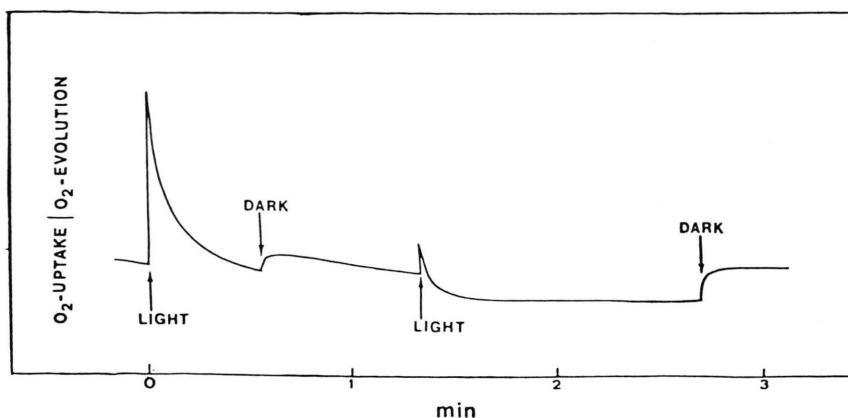
(Fig. 1 a, b) in order to produce the extent of evolution shown in Fig. 1 c. Also, the O_2-uptake clearly visible already at the onset of the illumination period is better seen after such a preillumination or the repeated illuminations used in Fig. 1 a, b and c.

Protoplasts without the bicarbonate addition do not show a steady state oxygen evolution. Instead, an oxygen gush is observed at the onset of illumination followed by a clear uptake (Fig. 2). The uptake persists in subsequent illumination periods whereas the gush is fully exhausted after two or three illuminations (Fig. 2). The gush is sensitive to DCMU whereas the uptake is not. From this it follows that the uptake is to be associated with photosystem I.

Protoplasts of the tobacco aurea mutant Su/su [11]. Leaves of this type of tobacco exhibit a photorespiration level which is many fold higher than in the wild type as described in many reports by different laboratories [12, 14]. Protoplasts of this type of tobacco show in the absence of bicarbonate in the reaction medium only the O_2-uptake and no gush (Fig. 3). On the basis of chlorophyll this uptake is 15 to 20 fold higher and on the basis of the chloroplast number (measured with a Coulter Counter according to ref. 7) or protoplast number higher by a factor of 4 to 5 when compared to that of the wild type. This obvious hangover of an oxidative process can only be due to a much higher content of an enzyme using oxygen as a substrate or a deviation of photosynthetic electron transport on the acceptor side of photosystem I in which either a higher endogenous concentration of the terminal auto-oxidisable electron acceptor is present [10] or simply another type of acceptor than in the wild type. Mass spectrometry shows that ^{18}O is taken up at a very rapid rate with

these protoplast preparations (Gerster and Schmid in preparation).

Protoplasts prepared from yellow leaf sections of the variegated tobacco mutant N. tabacum var. NC 95. Protoplasts of this tobacco mutant show essentially only a light-induced O_2-uptake (Fig. 4). At the onset of the first illumination sometimes a trace of an oxygen-gush is visible (Fig. 4). As is known from the literature [9, 15] these leaf sections exhibit only photosystem I-activity which again leads to the conse-
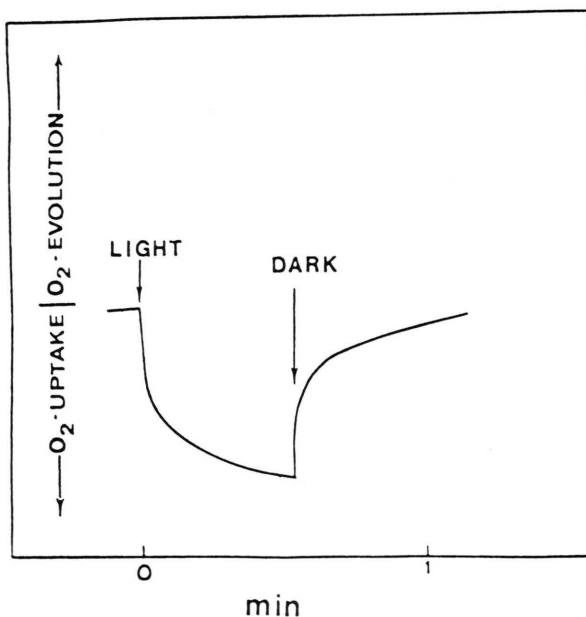
Fig. 3. Oxygen gas exchange in protoplasts of the tobacco aurea mutant *N. tabacum var. Su/su* without bicarbonate addition. Otherwise conditions as in Fig. 2. Chlorophyll content on the electrode 21 µg. Same electrode sensitivity as in Fig. 2.
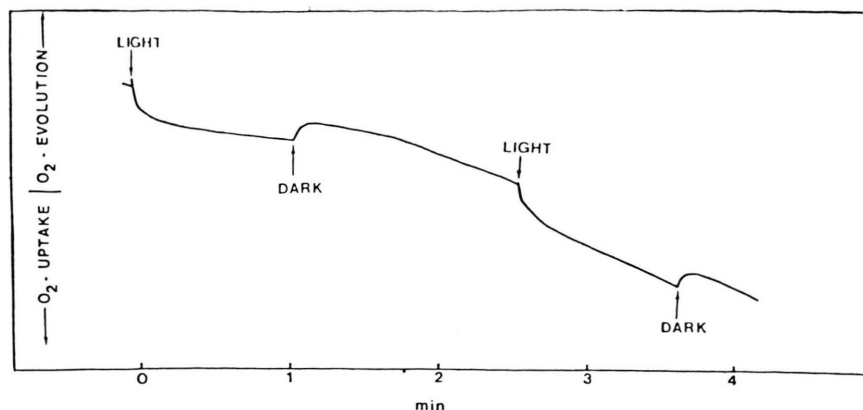
Fig. 4. Oxygen gas exchange in protoplasts prepared from yellow leaf sections of the variegated tobacco mutant *N. tabacum var. NC 95* without bicarbonate addition. Otherwise conditions as in Fig. 2 and 3. Chlorophyll content on the electrode 23 µg. Same electrode sensitivity as in Fig. 2 and 3.

quence that the light induced O_2-uptake has to be associated with photosystem I.

At this point it should be noted that in comparison to the protoplast preparations described in Fig. 1–4 intact chloroplasts prepared according to Homann and Schmid [9] show a completely different behavior. Thus, chloroplasts from wild type tobacco, as an example, show first an important oxygen gush
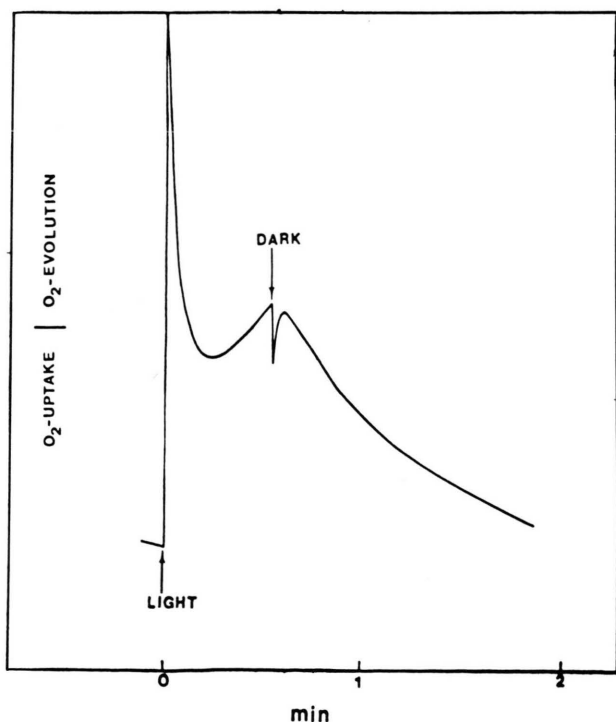

Fig. 5. Oxygen gas exchange in intact chloroplasts of the wild type tobacco without additions to the suspension medium. The chlorophyll content of the 1 ml suspension applied to the electrode was 56 µg.

followed by a steady-state oxygen evolution (Fig. 5). Interesting in such experiments are oxygen transients seen with such chloroplast preparations in the dark after the illumination period (Fig. 5). At first there is a fast uptake followed by an evolution. In the recent report by Schmid and Thibault [10] the photosynthetic oxygen-evolution in the flash sequence which shows the known oscillations, works entirely on the first oxygen gush seen in figure 5 with the rapid uptake described there being also contained in this gush. The fast uptake of Schmid and Thibault [10] ($\sim$25 msec in the mutant and 40 msec in the green control) obviously shows up here after switching off the light (Fig. 5). On the other hand the oxygen uptake described for protoplasts in this paper is a steady state phenomenon which persists over minutes if not hours and therefore is free of any induction phenomenon.

Action spectrum of the light-induced oxygen-uptake in tobacco protoplasts. The question concerning the photoreceptor for light-induced oxygen uptakes in biological systems is an ever returning problem and the subject of many discussions in the literature. Kowallik and Gaffron [16, 17] and Pickett and French [18] reported on a light-induced oxygen-uptake in green *Chlorella* and a chlorophyll-free but carotenoid-containing *Chlorella* mutant [16, 17]. Measurement of the action spectrum for the oxygen-uptake in the chlorophyll-free mutant led to a spectrum which did not allow the decision whether a carotenoid or a flavin would be the photoreceptor. At any rate a yellow pigment was the apparent condidate for the photoreceptor. Later Schmid and Schwarze [19] observed in a colorless *Chlorella* mutant which contained neither carotenoids nor chlorophyll essentially the same or at least a comparable uptake as
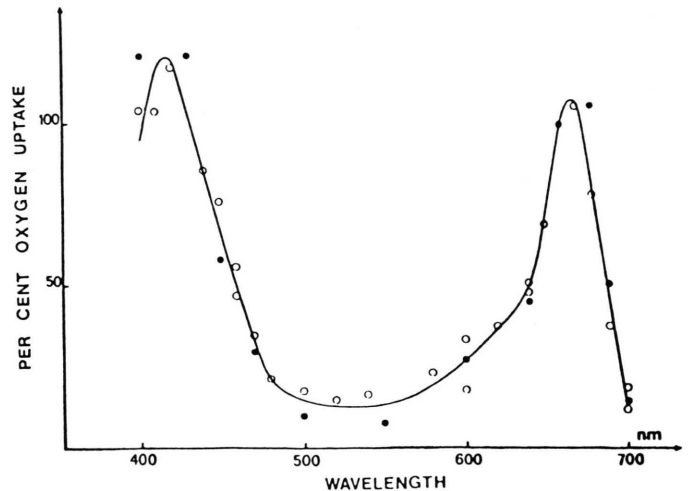
Fig. 6. Action spectrum for the oxygen-uptake in protoplasts of the tobacco *aurea* mutants *Su/su* (○) and *Su/su var. aurea* (●).

that observed by Kowallik and Gaffron [16]. In the case of the colorless *Chlorella* mutant, however, FMN was detectable in the mutant and was even excreted under certain conditions. As the oxygen-uptake was, as with the *Chlorella* mutant of Kowallik, essentially a blue light effect we proposed that a flavin should be the photoreceptor [19].

In the present case of the tobacco protoplasts the uptake is certainly not comparable or related to the oxygen-uptake reported by Kowallik and Gaffron [16]; Pickett and French [18] or Schmid and Schwarze [19] because the photoreceptor for the oxygen-uptake in protoplasts is clearly chlorophyll and no yellow pigment. The action spectrum for the light induced oxygen uptake in protoplasts is the spectrum of chlorophyll (Fig. 6). It is most astonishing that there is apparently no influence of carotenoids on this uptake. This is concluded from the following observation: our tobacco mutants *Su/su* and *Su/su var. aurea* differ considerably from the wild type tobacco with respect to their carotenoid content [6], with the *Su/su var. aurea* mutant having for example a four fold higher carotenoid content per chlorophyll present than the wild type. Nevertheless, protoplasts from the wild type, the two mentioned *aurea* mutants and protoplasts from yellow leaf sections of the variegated tobacco *NC 95* show the same wavelength dependence of the oxygen-uptake (Fig. 6). No influence of yellow pigments *e. g.* carotenoids is seen and any type of energy transfer from carotenoids to chlorophyll in the photosystem I unit for example can also be excluded.
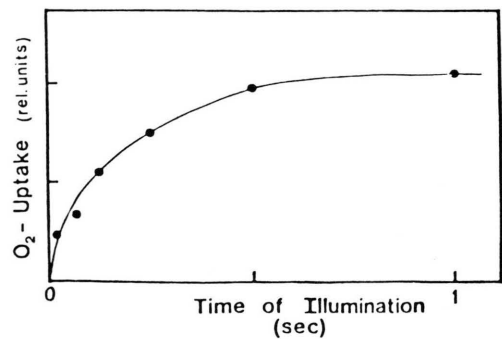

Fig. 7. Dependence of the light-induced oxygen-uptake on the illumination time in tobacco protoplasts at room temperature.

The dependence of the oxygen-uptake on the time of illumination shows that the reaction is rather rapid, requiring approximately 500 msec of illumination to be maximal (Fig. 7).

Discussion

In the present paper we have tried to qualitatively characterize the function of tobacco protoplasts with respect to their oxygen gas exchange in the light. From this investigation it appeared that protoplasts behave entirely different when compared to chloroplasts. Protoplast suspensions without the addition of bicarbonate never showed any sign of apparent steady-state oxygen-evolution. A glance on the oxygen tracing of the oxygen electrode was sufficient to

say whether the protoplast preparation had suffered an osmotic shock. Thus, protoplasts offer in principle the unique opportunity to study directly photorespiratory oxygen-uptake. At this point it should be borne in mind that in systems in which photosynthetic oxygen-evolution counteracts the uptake, e. g. leaves or entire plants, the only solid method to check for photorespiration is to follow ^{18}O-uptake by mass spectrometry [20], a practice used since years in this laboratory.

As has been said above, modern plant breeding uses such methods as protoplast fusion [4] or transfer of genetic material from one cell organel to another [5] and we, therefore, asked the question wheter protoplasts before a genetical manipulation could be tested functionally in a simple way. This is not a trivial task since protoplast are very fragile in many respects and cannot be agitated in the assay. However, the test appears quite feasable with our electrode system [10] now, if the question to be answered is, whether protoplasts have a high or low photorespiratory oxygen-uptake (Fig. 2, 4). Furthermore, in the course of these investigations it appeared what with protoplasts penetration problems with various substances can be easily overcome provided the concentration of the substance to be introduced into the protoplast is sufficiently high in the suspension medium. Thus, the assay system using protoplasts from the above tobacco mutants which differ so much with respect to their photorespiration should permit new insights into the mechanism of photorespiration, above all when applied to such shophisticated methods as mass spectrometry and others.

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