

# Isolation and Separation of Epidermal and Mesophyll Protoplasts from Rye Primary Leaves – Tissue-Specific Characteristics of Secondary Phenolic Product Accumulation

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Z. Naturforsch. **41c**, 22–27 (1986); received June 10, 1985

*Dedicated to Professor Hans Grisebach on the occasion of his 60th birthday*

*Secale cereale* L., Epidermal, Mesophyll Protoplasts, Localization of Flavonoids

We have developed a technique for the large-scale isolation of epidermal and mesophyll protoplasts, as well as the vascular strands, of rye primary leaf blades.

Separation of the two types of protoplasts has been successful only from leaves harvested at the end of a 13-h light period, when chloroplasts were enriched in starch.

The occurrence of different flavonoid compounds, and amounts, in epidermal and mesophyll protoplasts can be used as criteria for protoplast purity and viability since C-glucosylflavone O-glycosides are characteristic of epidermal protoplasts whereas flavone O-glucuronides and anthocyanins are typical of mesophyll protoplasts. Several non-flavonoid phenolic compounds are found only in the epidermal protoplast. These patterns of secondary product accumulation reflect the high tissue specificity of the rye leaf.

## Introduction

Protoplasts, naked plant cells, provide a unique experimental system that is very useful for studying cell structure, somatic cell genetics, subcellular compartmentation and many other aspects of cell physiology and biochemistry (for review see [1, 2]). Although a wide range of plant tissues have been used for isolating protoplasts, most research to date has been carried out with protoplasts derived from leaf mesophyll or from suspension cultures.

In our continuing program on the compartmentation of secondary plant products and their metabolism at the tissue and cellular level, especially of phenylpropanoid and flavonoid compounds, we have found mesophyll protoplasts isolated from developing primary leaves of oats and rye useful tools for localization studies [3–5]. Kojima *et al.* [6] were able to separate mesophyll and epidermal protoplasts from primary leaves of *Sorghum* and used these to investigate the location and metabolism of the cyanogenic glycoside dhurrin [6, 7]. Similarly, both types of protoplasts isolated from maize leaves were analysed [8]. Recently, we reported for the first time the occurrence of flavonoid compounds in isolated guard cell protoplasts from *Vicia faba* leaves [9]. In the present paper we report the simultaneous isolation and separation of epidermal and mesophyll protoplasts from rye primary leaves.

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## Materials and Methods

### Plant material

Rye seedlings (*Secale cereale* L. var. Kustro, purchased from F. von Lochow-Petkus, Bergen FRG) were grown in a phytotron as previously described [5]. 4 to 5-day-old primary leaves were harvested at the end of a 13-h light period when chloroplasts contained a high amount of starch (see below).

### Protoplast isolation and separation

For the simultaneous isolation of both types of protoplasts, the leaf epidermis (leaf length 5 to 7 cm) was removed by mechanical peeling and ca. 30 leaf blades were preplasmolyzed in a petri dish 9 cm in diameter with 0.6 M sorbitol as described elsewhere [3–5]. The osmoticum was replaced by the enzyme medium (a), 10 ml per dish (1.5% cellulysin grade B., Calbiochem Co, La Jolla, California, USA, in 5 mM MES/KOH buffer pH 5.8, containing 0.6 M sorbitol). After 1.5 h of incubation at 25 °C in dim light (1200 lux) without shaking, most of the yet-undigested epidermis (with some mesophyll adhering) was carefully transferred to 8 ml of fresh digestion medium. Mesophyll protoplasts were collected by centrifugation at  $50 \times g$  15 min and washed three times by resuspending the pellet in 0.6 M sorbitol

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Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen  
0341–0382/86/0100–0022 \$ 01.30/0



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containing 5 mM MES/KOH buffer pH 5.8 (medium b). The resulting suspension was essentially free from contaminating epidermal protoplasts, chloroplasts and other cell debris and a cushion centrifugation was not required. The protoplast yield was estimated to be approx. 70% based on recovery of chlorophyll from purified protoplasts. After an additional 1 h of incubation, epidermal and remaining mesophyll protoplasts were released from the undigested epidermal tissue. This digest was passed through a stainless steel screen to remove undigested material. Most of the contaminating mesophyll protoplasts were collected by centrifugation as described above. The supernatant was strongly enriched in epidermal protoplasts. This supernatant was mixed with the same volume of 5 mM MES/KOH buffer pH 5.8, 0.6 M sorbitol, containing 20% Ficoll (Ficoll 400, Pharmacia Fine Chemicals, Uppsala, Sweden), final concentration 10%, and poured into glass ampoules (Fig. 1). Afterwards, 1 ml of resuspension medium

#### Flavonol extraction and quantitation

Flavonoids were extracted from the protoplasts essentially as described in [5]. Liquid chromatographic separation was performed on a Lichrosorb RP-8 column (5  $\mu$ m, 250  $\times$  4 mm, MERCK, Darmstadt, FRG). 20  $\mu$ l of extracts, diluted with water to give 40% MeOH, were injected in the column, developed linearly within 25 min from 20% solvent B (1% phosphoric acid, 20% HOAc, and 25% acetonitrile in water containing 1 mM LiDS) in solvent A (1% phosphoric acid in water with 1 mM LiDS) to solvent B alone, at a flow rate of 2 ml min<sup>-1</sup>. Flavones, some non-flavonoid compounds and anthocyanins were detected at 340 and 520 nm, respectively. For quantitation, the HPLC system, detector and computing integrator see [11].

## Results and Discussion

### General characteristics of protoplasts isolated from rye tissues

Digestion of 4 to 5-day-old rye primary leaf blades as described under Materials and Methods yielded large numbers (4 to 5  $\times 10^6$  per g tissue) of mesophyll protoplasts with a yield of approx. 70%. Mesophyll protoplast diameter ranged from 25 to 35  $\mu$ m, in average; they contained one central vacuole with variable amounts of anthocyanin and chloroplasts when viewed under the microscope (Fig. 2). As seen in free-hand cross sections of leaf blades, subepidermal mesophyll layers contained the highest vacuolar anthocyanin concentrations while cells towards the innermost layer had less anthocyanin (data not shown). Mesophyll protoplast suspensions were essentially free of contaminating epidermal protoplasts (see below).

The epidermal protoplasts, simultaneously isolated from the leaf blades by flotation (Fig. 2), were similar to, or larger in diameter than mesophyll protoplasts, on the order of 20 to 60  $\mu$ m. The epidermal protoplasts do not contain anthocyanins or visible plastids. The yield of epidermal protoplasts was lower, ranging from 7 to 8  $\times 10^4$  per g tissue, and recovery was about 30% of the total number of parent epidermal cells as counted microscopically. Cross-contamination with mesophyll protoplasts amounted to approx. 5% or less.

Bundle sheath strands are resistant to the action of cellulysin and were completely separated from the

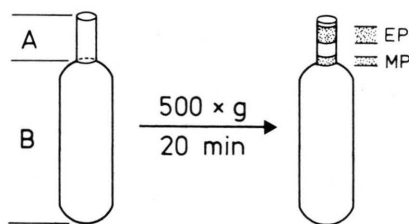


Fig. 1. Separation of epidermal (EP) and mesophyll protoplasts (MP) by flotation. A = 1 ml medium b; B = 38 ml of 10% Ficoll in medium b containing isolated rye protoplasts (c.f. Materials and Methods).

(b) was carefully put on top of the diluted suspension and the sample centrifuged at 500  $\times$  g for 20 min. During this step epidermal protoplasts floated to the upper zone of the medium (Fig. 1) while mesophyll protoplasts and debris remained at the surface of the Ficoll phase. To locate the protoplasts, 1 to 2 drops of neutral red in isolating medium were added to the suspension [6] before adding Ficoll. The dye was quickly taken up by the epidermal protoplasts and accumulated in their vacuoles. Epidermal protoplasts were collected and counted microscopically using a hemocytometer. Protoplast concentration was adjusted to approx. 0.5  $\times 10^6$  per ml. Chlorophyll content of leaf pieces and protoplasts was estimated according to [10].

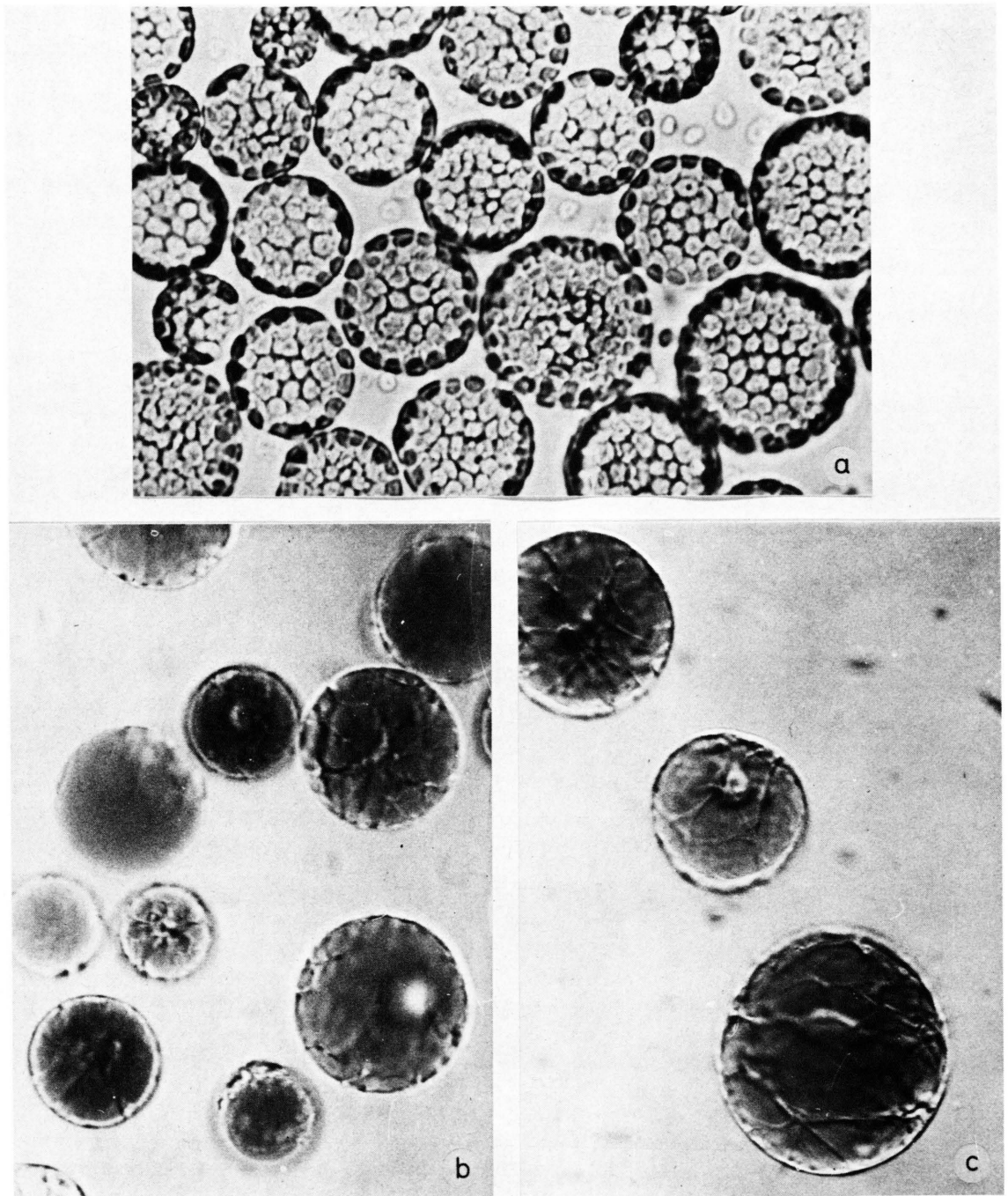


Fig. 2. Micrographs of protoplasts isolated from rye primary leaves (a): mesophyll protoplasts in transmittent light ( $\times 400$ ); (b, c): epidermal protoplasts, Nomarski interference contrast ( $\times 400$ ), stained with neutral red.



neighbouring mesophyll cells. These strands did not contain anthocyanins or any other flavonoid (data not shown).

Both types of protoplasts were stable over a 3-h period, exhibiting no significant loss in number when kept at 0 to 4 °C in resuspension medium. They remained viable and showed no visible clumping. Staining with neutral red, together with the exclusion of Evan's Blue [12], indicated the absence of non-viable protoplasts in the suspension. Furthermore, protoplasts retained their specific flavonoid products as estimated by comparison with the parent leaf tissues on a per cell and chlorophyll basis, respectively.

In our earlier work viable mesophyll protoplasts were successfully isolated in high yield (up to 90%) from oat and rye primary leaves of different developmental stages [3–5]. These leaves were harvested at the end of the dark phase of a 11/13-h photoperiod; however, they proved to be disadvantageous for a simultaneous separation of epidermal protoplasts by our flotation techniques. In that case, chloroplasts in the mesophyll protoplasts lacked starch, and mesophyll and epidermal protoplasts had similar densities, resulting in cross-contaminations of 50% or more. Several methods to separate these two types of protoplasts were unsuccessful (*c.f.* [6]). However, when leaves were harvested at the end of the light period, when starch was stored within the chloroplasts, mesophyll protoplasts were easily sedimented and separated from epidermal protoplasts.

#### *Different flavonoid compounds in epidermal and mesophyll protoplasts*

Fig. 3 shows the distribution of characteristic flavonoids in epidermal and mesophyll protoplasts isolated from rye primary leaves. The structures of these compounds have been previously reported [5, 13, 14].

As determined by HPLC, epidermal protoplasts exhibit two C-glucosylflavone O-glycosides, isovitexin 2''-O-arabinoside ( $R_3$ ) and isovitexin 2''-O-galactoside ( $R_4$ ). Mesophyll protoplasts contain two flavone O-glucuronides, luteolin 7-O-glucuronosyl-glucuronide 4'-O-glucuronide ( $R_1$ ) and luteolin 7-O-glucuronosyl-glucuronide ( $R_2$ ), as well as the anthocyanins, cyanidin 3-O-glucoside ( $R_I$ ) and cyanidin 3-O-diglycoside ( $R_{II}$ ) (Fig. 4). When the total flavonoid content is calculated (sum of components) for each type of protoplast, epidermal protoplasts contain approx. 90 nmol of  $R_3$  plus  $R_4$  /  $10^6$  individu-

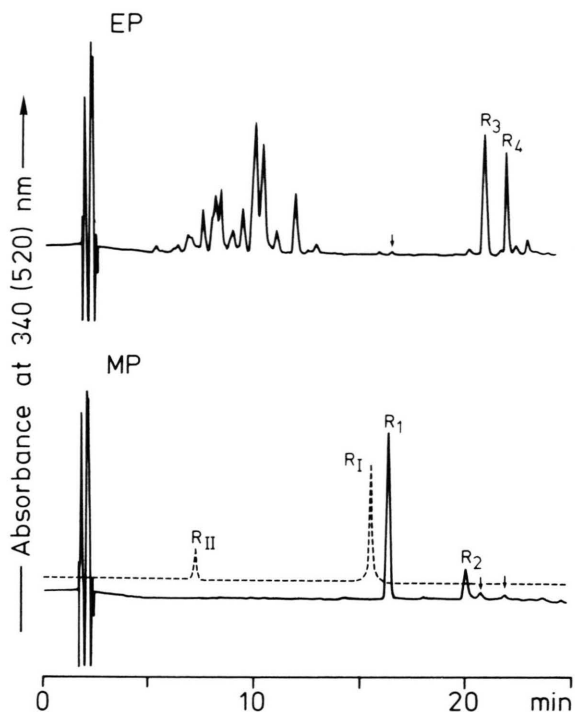


Fig. 3. HPLC profiles of methanolic extracts of epidermal (EP, upper screen) and mesophyll protoplasts (MP, lower screen) from rye primary leaves. Flavonoids  $R_1$  through  $R_4$ , detection wavelength 340 nm;  $R_I$  and  $R_{II}$ , 520 nm. Unidentified early peaks obtained with EP (340 nm) represent simpler phenolic compounds such as hydroxycinnamic acid conjugates. Protoplast concentrations: EP  $0.54 \times 10^6$ /ml; MP  $0.51 \times 10^6$ /ml; 20  $\mu$ l of each extract were subjected to HPLC.

als whereas mesophyll protoplasts contain 80 nmol of luteolin glucuronides  $R_1$  and  $R_2$ , and 40 nmol of cyanidin glycosides  $R_I$  and  $R_{II}$ .

These results confirm our earlier findings [5] of tissue-specific locations of luteolin glucuronides ( $R_1$ ,  $R_2$ ) and cyanidin glycosides ( $R_I$ ,  $R_{II}$ ) in the mesophyll of rye primary leaves. However, mesophyll protoplast preparations used earlier were reported to contain compounds  $R_3$  and  $R_4$  in significant amounts. Using improved techniques for the simultaneous isolation of epidermal and mesophyll protoplasts, we now conclude that C-glucosylflavone O-glycosides ( $R_3$ ,  $R_4$ ) were contaminants from some epidermal protoplasts present in the earlier mesophyll preparations [5]. With the methods described in this paper we were able to prepare mesophyll protoplasts without significant cross-con-

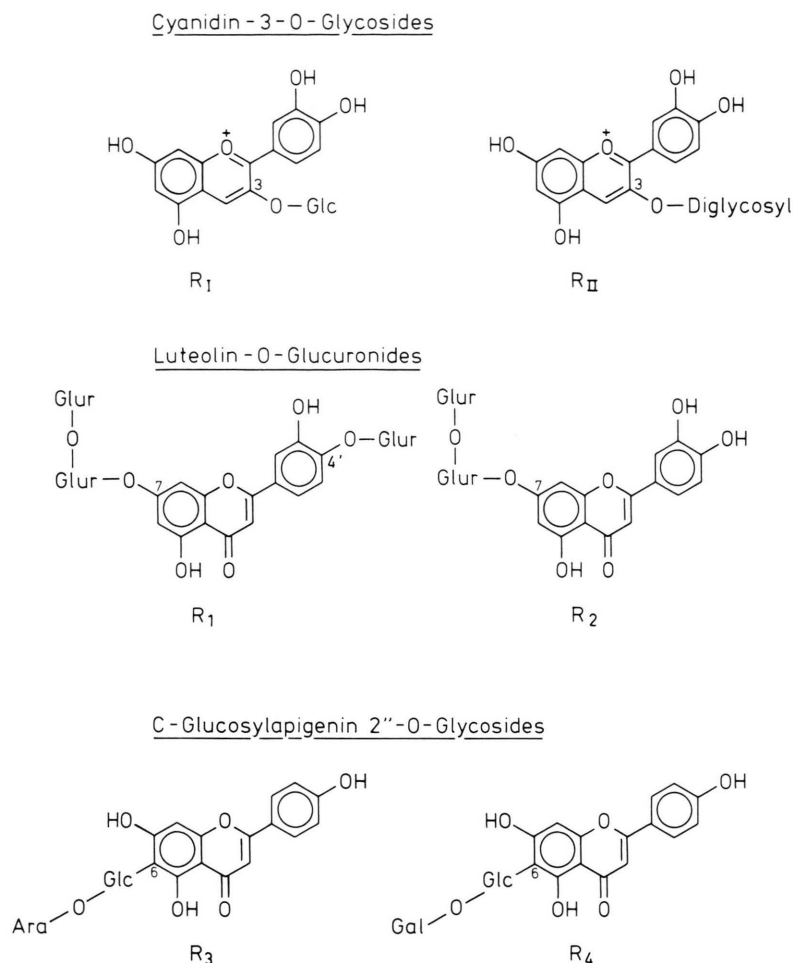


Fig. 4. Flavonoids of rye primary leaves and protoplasts (see Results and Discussion).

tamination by epidermal ones and find that the epidermal flavones  $R_3$  and  $R_4$  are below their detection limit (Fig. 3). A 1.5-h digestion of leaf blades with 1.5% of cellulysin instead of 3 to 4 h with 2% [5] leads to the removal of most of the mesophyll protoplasts leaving epidermal cells totally intact. On the other hand, neither purified epidermal protoplasts nor epidermal peels showed appreciable contamination by mesophyll flavonoids (*c.f.* [5]).

In addition to flavonoids other phenolic substances of unknown structure were present in epidermal preparations (Fig. 3; [5]). These substances are probably hydroxycinnamic conjugates based on their primary absorptivity at 290 to 320 nm and by their fluorescence behaviour on TLC plates. Structural elucidation is in progress.

## Conclusion

These techniques allow the simultaneous large-scale isolation of protoplasts from the photoautotrophic mesophyll and the heterotrophic epidermis of rye primary leaves. Furthermore, flavonoid amounts as well as distribution patterns can be used as suitable criteria to prove protoplast purity and viability. However, it is important to realize that flavonoid contents and patterns change during leaf development: During the first five days of growth all flavonoids accumulate rapidly; afterwards, epidermal flavonoids  $R_3$  and  $R_4$  remain at a constant level whereas mesophyll components, luteolin and cyanidin derivatives  $R_1$  and  $R_{II}$ , decrease continuously [5].

Further investigations will be necessary to elucidate whether mesophyll and epidermal tissues are

autonomous in the production of their flavonoids, or whether an intercellular transport of secondary compounds or precursors occurs, *e.g.* from mesophyll to epidermis (*c.f.* [15]).

#### Acknowledgements

We gratefully acknowledge the financial support from the "Deutsche Forschungsgemeinschaft" and

the "Minister für Wissenschaft und Forschung des Landes Nordrhein-Westfalen". The authors are grateful to Andreas Peters for his help in protoplast preparation, Prof. K. Schmitz and Dr. D. Strack, Köln, for their help in microphotography and HPLC analysis, respectively.

We thank Prof. J. W. McClure, Oxford/USA, for revising the English version of the manuscript.

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