"Flavanone Synthase" in Oat Primary Leaves
Time Course and Distribution at the Tissue and Subcellular Level

Klaus Fuisting and Gottfried Weissenböck

Botanisches Institut der Universität zu Köln, Gyrhofstr. 15, D-5000 Köln 41

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Oat Primary Leaves, "Flavanone Synthase", Time Course, Localization

"Flavanone Synthase" (FS) isolated from oat primary leaves shows maximal activity in the differentiating zone of five day old leaves. Activity of FS is distributed in both the upper and lower epidermis, but dominant in the mesophyll. After fractionation of mesophyll protoplasts of *Avena* by differential centrifugation, FS activity could only be detected in the "cytosol" and not in intact chloroplasts or other particulate fractions.

Introduction

The dynamics and distribution of C-glycosylflavones in developing primary leaves of oats has been studied by Effertz and Weissenböck [1, 2]. They showed that flavones accumulate maximally in five to six day old leaves in both the epidermal tissues and in the mesophyll from phytotron-grown seedlings. Thus oat primary leaves differ from *Sinapis alba* cotyledons [3, 4], and onion scales [5, 6], where flavone accumulation and metabolism takes place mainly in the epidermis.

In oat primary leaves the enzyme phenylalanine ammonia-lyase (PAL), which initiates the phenylpropanoid pathway, exhibits its highest activity in five to six day old primary leaves too [7]. The distribution of PAL activity in epidermal layers and in the mesophyll coincides with the levels of flavonoids accumulating in these tissues. However chalcone isomerase (CI) activity does not follow the time course of PAL activity and is restricted almost entirely to the mesophyll [7].

To extend our knowledge of distribution and kinetics of flavonoid metabolism in oats it is necessary to investigate the "flavanone synthase" reaction, which forms naringenin, the possible C-15 precursor of all other classes of flavonoids [8]. It has recently become evident that this reaction consists of the enzymatic synthesis of the chalcone followed by either autocyclisation or by enzymatic cyclisation of the chalcone [9, 10]. In the present paper the overall reaction is called "flavanone synthase" reaction (FS).

Reprint requests to Prof. G. Weissenböck.

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Little is known about the tissue or intracellular localisation of FS [11–14]. Whereas Ranjeva *et al.* [13] found that chloroplasts contained more than 50% of the FS activity of *Petunia* leaves, Hrazdina could not detect FS activity in chloroplasts isolated of different plants including *Brassica, Pisum* and others [12].

In *Avena* approximately 10% of the PAL and 20% of the CI activity is found in chloroplasts isolated from primary leaves [15]. Furthermore oat chloroplasts contain a characteristic assemblage of C-glycosylflavones [16].

Based on these informations the present investigation was designed to measure the FS in leaf sections, tissues, and subcellular fractions derived either from the whole leaf or from protoplasts, to determine the potential for flavonoid synthesis in these compartments.

Materials and Methods

Plant material: Avena sativa L., *cv.* Gelbhafer Flämingskrone, was grown under our standard conditions in a phytotron [17]. Four to eight day old seedlings were used to study enzyme activity as a function of leaf development. Tissue distribution, protoplast, and chloroplast experiments were done on five day old primary leaves.

Chemicals: *p*-coumaroyl CoA was either a gift of G. Hrazdina, Geneva, NY, or synthesized according to [18]. [2-^{14}C]malonyl-CoA (37 mCi/mmol) was purchased from NEN, Boston, Mass.

Preparation of leaf sections and tissues: following our earlier technique [7] primary leaves were cut into three sections: a 1 cm basal, highly meristematic, chlorophyll deficient section, which is en-

closed within the coleoptile of the intact seedling, a 1 cm apical section, and the middle section which increases in length as the leaf matures. To study tissue distribution of the FS, the epidermis was carefully peeled from either the lower or upper side of the middle section of the leaf. Epidermal layer and the complementary part of the leaf consisting of mesophyll and the remaining epidermis were then analyzed. Experiments were repeated at least twice using 100 seedlings per sample for the youngest stage of development and about twenty seedlings for the latest stages or for tissue distribution studies.

Protoplast and organelle isolation: Photosynthetically active mesophyll protoplasts were prepared and ruptured mechanically according to [19]. Subcellular fractions were separated by differential centrifugation of the ruptured protoplasts for 90 seconds at $1000\times g$ (chloroplast fraction, checked for envelope intactness according to [20]), 15 minutes at $10000\times g$ (mitochondrial fraction), 30 minutes at $170000\times g$ ("microsomal pellet" resp. soluble cytosol). Pelleted fractions were resuspended in the protoplast wash medium before the enzyme assay. Chloroplasts from whole leaf extracts were prepared according to [21]. Enzyme preparation: All steps were carried out at 4 °C. Leaves were homogenized under liquid nitrogen, and the nitrogen powder extracted with five volumes (v/w) of 200 mM potassium phosphate buffer, pH 8, containing 5 mM mercaptoethanol, 10% (w/w) of both insoluble PVP and Dowex 1X2 (Cl^- form). After stirring for twenty minutes, particulate debris was removed by centrifugation for twenty minutes at $40000\times g$. An aliquot of the supernatant was passed through a 10 ml Sephadex G 25 column, which had been equilibrated with the extraction buffer. The protein was eluated in 3 ml of buffer. For tissue distribution experiments the G 25 step was omitted. Protoplasts and subcellular fractions were incubated directly in their corresponding suspension medium buffered to pH 7.9.

Flavanone Synthase Activity: FS was measured by a procedure modified from [12, 22]. The assay consisted of 50 µl protein solution, 50 µl of 200 mM potassium phosphate buffer pH 8, containing 4 mg/ml BSA, 5 µl ascorbic acid (5 µmol), 5 µl *p*-coumaroyl-CoA (0.9 nmol), and 5 µl [2-^{14}C]malonyl-CoA (1,35 nmol). After incubation for 20 minutes at 30 °C, the reaction was stopped with 50 µl methanol/acetic acid (1:1/v/v) which contained 1 mg/ml naringenin (Fa. Roth, Karlsruhe) as "carrier". The mixture was shaken vigorously with 200 µl ethylacetate [23], and after a centrifugation step, 100 µl of the upper phase was chromatographed in benzene/ethylacetate/methanol/water (upper phase 6:4:1:3) on precoated silicagel TLC plates (Merck, Darmstadt). The spot corresponding to naringenin was scrapped off into a scintillation vial, dissolved in 5 ml methanol, and radioactivity measured in a Packard liquid scintillation counter with a PPO/POPOP/toluene cocktail (5 g/0.3 g/1 l). Radiochemical purity of the reaction product in the standard system was monitored by cochromatography with authentic naringenin in the following systems:

toluene/ethylformiate/formic acid (5:4:1) [24] on precoated silica gel TLC plates, 15% ethanol, 20% ethanol, and benzene/acetic acid/water (115:72:3) [25] on cellulose TLC plates (Macherey und Nagel, Düren). In all of these systems the naringenin derived form the standard chromatograms was more than 90% radiochemically pure. When the enzyme preparations were denatured by boiling or by using trichloroacetic acid (TCA) and in assays without *p*-coumaroyl-CoA, no radioactivity was detected in the region corresponding to naringenin.

Protein and chlorophyll determination: After precipitation with TCA protein was dissolved in sodium dodecylsulfate (SDS) and determined according to [26]. Chlorophyll was determined by the method of Kirk [27].

Results and Discussion

Properties of the FS reaction in oats

Enzyme activity was linear with time up to 20 minutes and with protein concentration up to at least 50 µg/assay. Maximal enzyme activity was observed at pH 8 in potassium phosphate buffer. 50 mM Tris-HCl buffer inhibited the enzyme activity about 50%, whereas 5 mM MES and 10 mM HEPES buffer, used for the isolation of protoplasts respectively chloroplasts, did not affect FS activity. Triton X100 up to 1% (w/v) in the assay medium did not influence naringenin formation. Release products of the reaction as described in [22, 28, 29] were not detected under our experimental conditions.

Kinetics of extractable FS activity in different leaf sections

FS activity in the oat primary leaf reaches its maximum in five day old seedlings (Fig. 1 a). At this

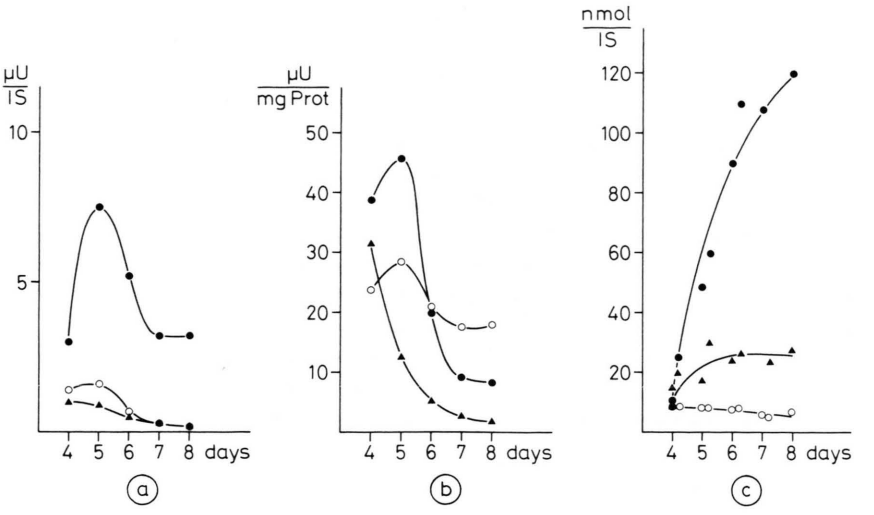

Fig.1. Kinetics of FS activity in oat primary leaves; a) activity per individual section, (▲–▲) top section, (●–●) middle section, (○–○) basal section; one unit is defined as one µmol naringenin formed per minute at 30 °C, data represent mean of three experiments; b) specific activities of FS in different leaf sections; c) kinetics of total flavone content in the different leaf sections, from [7].

stage highest specific activity is found in the middle section of the leaf (Fig. 1 b). Mixing experiments with leaves of different ages showed that FS activities were strictly additive.

At the fourth day after germination, after the leaf has penetrated the coleoptile the top section exhibits a relatively high FS activity, but decreases during the following period. During this time FS activity correlates well with flavone accumulation in this section (Fig. 1 c). In the same way, extractable FS activity in the middle section corresponds to the flavone accumulation curve in this region (Fig. 1 c).

Whereas the top and middle parts of the leaf show considerable changes in FS activity, the basal part remains nearly constant in both enzyme activity and flavone accumulation throughout the experimental period.

Furthermore it has to be mentioned that in the flavone poor [17] *Avena* coleoptile, no FS activity could be detected.

These observations indicate a coordinated development of FS activity and flavone accumulation in differentiating tissues of oats, similar to the pattern found in *Petroselinum* cell cultures [30]. Similar time courses of activity have been determined for PAL [7] and *p*-coumaroyl-CoA ligase [31] in oat primary leaves. But in contrast to the enzymes of phenylpropanoid metabolism investigated in *Petroselinum*, the enzymes described for *Avena* do not show significant time differences between the enzyme activities of group I and group II [30]. Furthermore our results show a pattern of phenolic differentiation which corresponds to morphological differentiation in the oat primary leaf.

Occurence of FS activity in different leaf tissues

To compare FS activities of epidermis and mesophyll, we chose the middle section of a five to six day old leaf. Epidermal tissues were peeled from the upper surface of ten leaves and from the lower surface of ten others. Extractable FS activity was measured in the epidermal preparations and the complementary part of the leaf. By this technique one can calculate enzyme activities in each epidermis and in the mesophyll [7]. Controls were performed with untreated leaves.

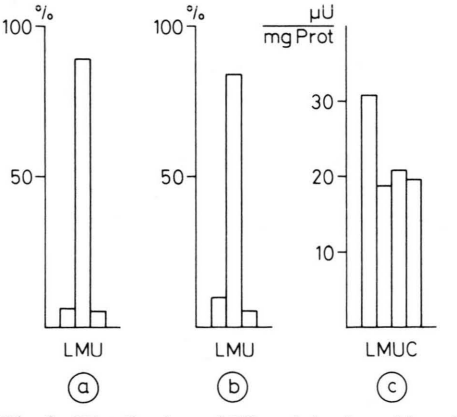

Fig. 2. Distribution of FS activity in epidermis and mesophyll; a) relative distribution of protein in upper (U), lower (L) epidermis, and in the mesophyll (M) of a five to six day old primary leaf; b) relative FS distribution; c) specific activities of FS ((C) control with unpeeled leaf).

More than 80% of the extractable protein and FS activity is localised in the mesophyll (Fig. 2). Comparing the specific activities, the upper epidermis and the mesophyll show similar levels, whereas the activity of the lower epidermis is about 30% higher. These data confirm the observation that flavonoid metabolism in oats is not restricted to the epidermis, as was reported for *Sinapis alba* but takes place to an appreciable amount in the mesophyll [2, 7]. While extractable enzyme activity may not reflect the *in vivo* activity, our investigations with *Avena* show clearly that the epidermis as well as the mesophyll has several enzyms which must be required for each tissue to be autonomous in flavonoid biosynthesis.

FS activity profile of protoplasts

When mesophyll protoplasts were prepared from oat leaves by the technique of Haas *et al.* [19], ruptured and fractionated by differential centrifugation, 95% of the chlorophyll and more than 60% of the protein is found in the 90 second chloroplast pellet (Fig. 3). These chloroplasts were at least 60% intact based on the permeability of the envelope to ferricyanide [20]. The activities of FS in the chloroplast enriched fraction and the other particulate fractions is minimal and after recentrifugation of the resuspended chloroplast pellet, FS activity could no longer be measured in this fraction. Sonification and the use of detergents, such as Triton X 100, Na-deoxycholate, and several others did not disclose FS activity in the chloroplast fraction. Variation of pH to pH 5.8 or incubation with DTT in the assay medium according to [32] did not activate FS in the chloroplast pellets. We obtained similar results with highly purified intact chloroplasts (90% intactness according to [20]) isolated by rapid centrifugation of leaf homogenates with a cleaning step over a Percoll gradient followed by two washings [21].

From these experiments we conclude that FS activity in mesophyll protoplasts isolated from oat leaves is a soluble, apparently "cytosolic" enzyme. Thus in contrast to the results of Ranjeva *et al.* [13], who found a high FS activity in *Petunia* chloroplasts, our results do not support the general hypothesis that the chloroplast is the dominant site of the synthesis of the flavanone skeleton.

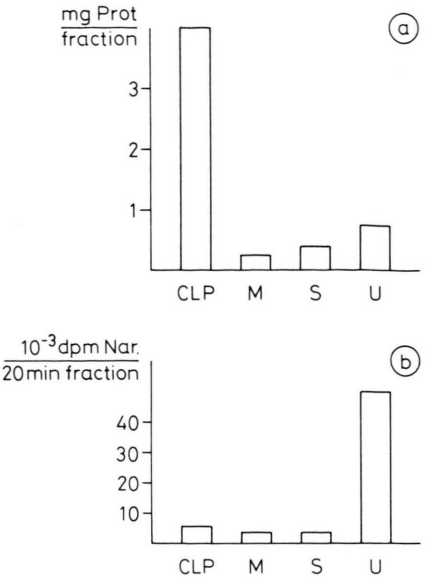

Fig. 3. Fractionation of oat mesophyll protoplasts by differential centrifugation; a) protein profile (Clp: chloroplast pellet, M: mitochondrial pellet, S: microsomal pellet, U: supernatant); b) FS activity profile (1000 dpm Nar ~ 0.23 μU).

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