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CHALCONE SYNTHASE IN THE LIVERWORT *MARCHANTIA POLYMORPHA*

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Key Word Index—*Marchantia polymorpha*; Marchantiaceae, liverwort; gametophyte; chalcone synthase.

Abstract—Chalcone synthase (CHS) activity was measured in gametophytic tissue of *Marchantia polymorpha*. The main product of the enzyme reaction was identified as naringenin which results from cyclization of the chalcone. Cross-reactivity under native and denaturing conditions was obtained using antibodies raised against CHS from three different higher plants, indicating the presence of CHS protein in *M. polymorpha*.

INTRODUCTION

Flavonoids are ubiquitous components of higher plants [1]. The key enzyme of flavonoid biosynthesis, chalcone synthase (CHS), catalyses the formation of 4,2′,4′,6′-tetrahydroxy-chalcone by condensation of one molecule of *p*-coumaroyl-CoA with three molecules of malonyl-CoA [2]. CHS protein sequence is highly conserved in higher plants and has a considerable similarity with stilbene synthase [3], an enzyme catalysing a related reaction.

Flavonoids (mainly flavones) are detectable in 40–50% of the investigated species of mosses and liverworts. In liverworts, flavone *C*- and *O*-glycosides, flavonols, dihydroflavones, dihydrochalcones and aurones have been found; mosses contain flavone *C*- and *O*-glycosides, biflavones, aurones, isoflavones and 3-deoxyanthocyanins [4]. Tricetin di-*C*-glycosides could serve as a chemical marker for liverworts and biflavonoids for mosses [5]. From the liverwort, *Marchantia polymorpha*, several flavonoids have been isolated comprising aurones, apigenin and luteolin *O*-β-D-glucuronides and other apigenin and luteolin *O*-glycosides [6]. However, it has to be considered that the most characteristic compounds of liverworts are bibenzyls [7], which could arise from a stilbene synthase type of reaction [8].

To date, biochemical investigations of phenylpropanoid metabolism including flavonoids in bryophytes are rare [9]. Characterization of the postulated CHS from a flavonoid-producing bryophyte would be an important step to allow the evolutionary comparison of vascular and lower plants. A detailed knowledge of the enzymology would reveal how flavonoid biosynthesis functions in lower plants and allow speculation on how such pathways resulting in flavonoid products may have evolved in the history of plants [8]. Prior to using flavonoids as chemical markers in chemotaxonomy, it is necessary to consider their biogenetic pathways [10].

The initial evidence for the occurrence of CHS in several flavonoid-containing bryophytes, including *M. polymorpha*, were recently obtained by testing for CHS activity in crude extracts and subsequent product analysis by TLC. Using the latter method, vegetative as well as reproductive (male and female gametophors) tissues of *M. polymorpha* have been investigated (Böttcher and Weissenböck, unpublished results). As a second step, we wished to verify the presence of CHS in *M. polymorpha* by identifying the resulting reaction product. Furthermore, the cross-reactivity of protein from *M. polymorpha* was investigated by using antibodies raised against CHS protein from three different higher plants.

RESULTS AND DISCUSSION

Extraction of protein and CHS assay

The extraction and assay procedure for CHS from gametophyte was developed according to the method for rye primary leaves [11]. Because of the continuous growth of the liverwort thallus by initial cell groups, it is not possible to ascertain the age or stage of development. Thus, there were variations in protein content between *ca* 1 and 2 mg g fr. wt^{-1} and in specific CHS activity between 0.4 and 0.6 pkat mg^{-1} protein (extraction at pH 6). Product analysis by TLC showed that the main product formed in the assay was naringenin (40–60% of total product) which resulted either from autocyclization of the unstable chalcone or the occurrence of chalcone isomerase which has not been further investigated. In

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addition, two other products which contained *ca* 30–50% (byproduct 1) and 10% (byproduct 2) of the radioactivity incorporated into EtOAc-extractable substances were detected. TLC on cellulose in 15% EtOH showed that the R_f of byproduct 1 was 0.81 (R_f of naringenin was 0.24) which corresponded with observations using a higher plant [12]. Byproduct 2 was not detectable in this solvent. The byproducts were not further analysed. The activity of a stilbene synthase, in particular resveratrol synthase, could be excluded by co-chromatography with an authentic sample of resveratrol on cellulose in chloroform–acetic acid (3:2:H_2O satd); R_f of resveratrol was 0.28. This result was confirmed by TLC in three other solvents.

For preparation of CHS from many higher plants, an extraction buffer of pH 8 is used [2, 11, 13–15]. In the case of *M. polymorpha*, the reduction of extraction buffer pH to 6 caused a 34% increase in enzyme activity. Ascorbate is probably more stable under these conditions [16], resulting in a better prevention of oxidation. A comparable observation has been made with CHS from *Juglans* sp. where an extraction buffer of pH 6.3 was used [17]. On the other hand, with *M. polymorpha* the amount of extracted protein at pH 6 was reduced to *ca* 50%. It is well documented in higher plants that the quantity of extractable proteins increases when the pH of extraction buffer is raised [18]. In spite of the reduction of extracted protein there was an increase in CHS activity at pH 6, as mentioned above. Therefore, the amount of extracted CHS is not affected by this reduction of proteins. The optimal concentration of K-Pi in the extraction buffer was in the range of 0.1–0.5 M. To keep the influence of the crude extract (pH 6.1) on the assay pH of 8 as small as possible, 0.1 M K-Pi was chosen. The concentration of Na ascorbate (31 mM) and L-cysteine (18 mM), as used for the CHS extraction of rye primary leaves [11], proved to be in the optimal range. The substitution of Na ascorbate and L-cysteine by 5 or 10 mM mercaptoethanol resulted in a 30% decrease of activity in both cases. Addition of 0.3% BSA to the extraction buffer had no influence on CHS activity.

HPLC measurements at 280 nm showed that the crude extract contained various phenolic compounds. Nevertheless, CHS activity was not affected by the addition of Dowex (Cl^-) or insoluble PVP, although they led to a strong reduction of these compounds in the extract. CHS activity decreased with concentrations exceeding 20% (Dowex) or 10% (PVP) of fr. wt. The reduction of the soluble phenolics by using Sephadex G 25 did not affect the CHS activity. The addition of 2% PEG to the extraction buffer resulted in an increase of CHS activity by 12%. PEG is also known as a phenol-complexing agent [19]. CHS from walnut tree tissue depended very strongly on the presence of PEG in the extraction buffer [17].

The assay was performed at pH 8 because lower values increased the formation of byproducts. L-Cysteine stabilized the synthesis of naringenin whereas other protecting agents in the assay (0.5–8 mM DTT, 10–50 mM Na ascorbate, 1–6 mM mercaptoethanol) increased the total activity but this was only due to an increase of byproduct 1 but not of naringenin. The ionic strength of the assay buffer was raised from 0.1 M K-Pi to 0.25 M because the yield of naringenin increased under these conditions. Addition of 2% BSA to the assay buffer led to a 90% higher enzyme activity.

The low protein content of *M. polymorpha* thalli made it necessary to concentrate the extracted protein by pptn for product identification of CHS activity as well as for immunodetection of the enzyme. Consequently, the extraction conditions had to be changed. For the extraction buffer, pH 8 was chosen to avoid acidification below pH 6 due to the addition of $(NH_4)_2SO_4$ for CHS pptn. There was a strong increase of phenolic compounds during the protein pptn step. In an effort to minimize this accumulation, insoluble PVP and Dowex (Cl^-) were added to the extraction buffer.

Identification of the reaction product

Due to the two byproducts, the first step was the separation of the EtOAc extracted products via HPLC (Fig. 1). The retention time of the major detectable peak,

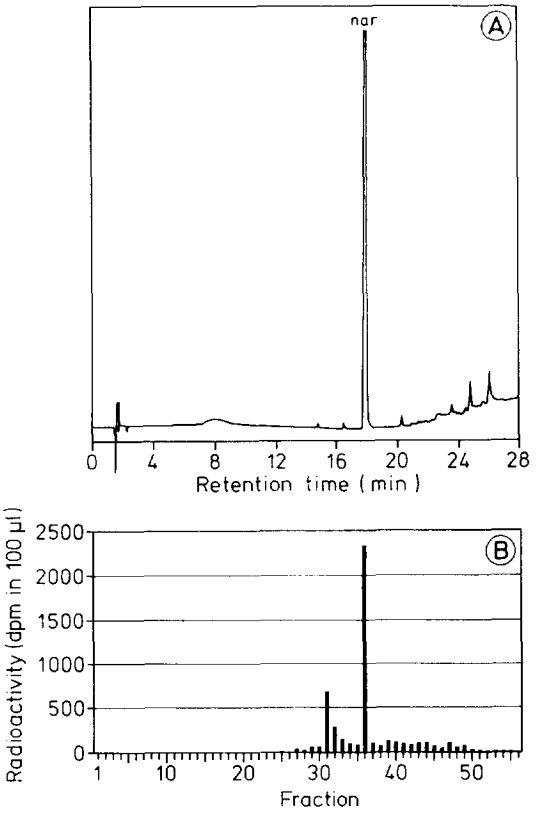

Fig. 1. HPLC analysis of EtOAc extractable CHS assay products. Before application to the C_8-column authentic naringenin was added to the sample. The eluent was collected by a fraction collector. (A) Elution profile, using a linear gradient from 0 to 100% acetonitrile in H_2O over 25 min and a flow rate of 1 ml min^{-1}.nar = Naringenin. (B) Amount of radioactivity in each fraction (fraction period = 30 sec). Fractions numbers 31, 36 and 39–42 were further analysed by TLC (see text).

belonging to authentic naringenin added to the sample before application to HPLC, corresponded to fraction 36 which contained 46% of the eluted radioactivity. TLC of this fraction on cellulose and silica gel plates in nine different solvent systems showed in all cases that the radioactively-labelled CHS reaction product behaved in the same way as the authentic naringenin used for co-chromatography (for R_f values see Experimental).

TLC of fraction 31 in chloroform–acetic acid (3:2:H_2O satd) showed that this product had the same R_f value (0.72) as byproduct 1. TLC of the 'background' radioactivity of fractions 39–42 in chloroform–acetic acid (3:2:H_2O satd) revealed that it corresponded to the second byproduct (R_f 0.97). This product was probably derived from [2-^{14}C]malonyl-CoA because it appeared also in control assays lacking *p*-coumaroyl-CoA or enzyme extract.

Immunodetection of CHS protein

The cross-reactivity of protein extracted from *M. polymorpha* was tested using antibodies against CHS from higher plants. The following abbreviations for these antibodies were used: Rm for the monoclonal anti-CHS (rye) antibody [20], Pp for the anti-CHS (parsley) serum [21], Sp for the polyclonal anti-CHS (spinach Al) antibody [22]. In all cases, samples of *Marchantia* crude extract at pH 8, the 0–50% ppt, the 50–70% ppt, $(NH_4)_2SO_4$ each, and crude extract from rye primary leaves [11] as control were applied to SDS–PAGE. The results are presented in Fig. 2. All antibodies recognized the rye CHS double band at M_r of 43 000 and 44 000 [20] which served as a positive control. Regarding *M. polymorpha* in the 50–70% $(NH_4)_2SO_4$ fraction, one distinct band with a M_r of about 46 000 was marked by Rm. As a further indication for immunodetection of CHS protein, the intensity of staining apparently corresponded to the measured enzyme activities. The band was very faint in crude extract samples and not detectable in the 0–50% ppt. The enzyme activity of crude extract was low because of the low protein concentration, the activity of the 50–70% ppt was five to six times higher with a three to four times higher protein content. The level of enzyme activity in the 0–50% ppt was even below the activity in crude extract. Moreover, applying protein directly extracted in sample buffer also resulted in the detection of a band with a M_r of *ca* 46 000. Using Pp for immunostaining, the signal at a M_r of 46 000 also appeared very faint in the crude extract and quite strong in the 50–70% ppt. Furthermore, there was an additional band showing a M_r of *ca* 77 000, quite strong in the crude extract and still more intense in the 50–70% ppt. It was absent in the 0–50% ppt. Because of the correspondence of enzyme activity and the appearance of this band with M_r of 77 000, it cannot be excluded that it is caused by a CHS or perhaps a degradation product of CHS of a higher M_r. However, there was no detection of this band by the other two antibodies. The Sp antibody was shown to be less specific when applied to rye as compared to the Rm and Pp antibodies. In addition to the double band of rye CHS, several other bands were marked. The signal with M_r 46 000 was also detected in the 50–70% ppt of *M. polymorpha*, in addition to some other weaker bands.

To investigate whether the antibodies obtained from higher plants also recognized the native protein responsible for CHS activity in *M. polymorpha*, immunoprecipitation was done using the 50–70% $(NH_4)_2SO_4$ protein fraction. With Pp diluted 1:2, *ca* 70% of the enzyme activity was precipitated (Fig. 3). In a related experiment using Sp in a dilution of 1:4, CHS activity was reduced *ca* 66% (data not shown). At a dilution of 1:256 activity was only halved, whereas this antibody showed no immunoprecipitation of spinach CHS at a dilution of 1:64 [22]. Both antibodies were raised against the respective native CHS proteins [21, 22]. Rm diluted 1:2, showed the lowest reduction of CHS activity (20 and 40% in two different experiments, data not shown). This might be due to the fact that this antibody was raised against SDS-denatured CHS protein [20].

In conclusion, to the best of our knowledge this is the first report on the occurrence of chalcone synthase in a bryophyte. The extraction and assay procedures were found to be similar to those of higher plants. There was no hint of the activity of stilbene synthase in the CHS assay using *p*-coumaroyl-CoA. On the other hand, the occurrence of lunularic acid in *M. polymorpha* [7, 23] suggests the presence of a bibenzyl synthase. However, its substrate seems to be dihydro-*p*-coumaric acid rather than *p*-coumaric acid itself [7]. SDS–PAGE, followed by immunoblotting, using three different antibodies from higher plants exhibited one distinct signal with M_r of 46 000 which is comparable to the values found for CHS subunits from most higher plants [e.g. 20–22, 24–25]. For the band with M_r 77 000, at present no clear interpretation can be made. Immunoprecipitation did not lead to complete reduction of activity and it can be speculated that this may be due to different isoforms of CHS with different immunogenicity to the antibodies used in the analyses. On the other hand, the incomplete precipitation may have a non-enzymatic origin or may be due to the presence of an enzyme competing for the same substrates.

The next step should be the purification of CHS from *M. polymorpha* to characterize this enzyme for comparison with higher plants regarding biochemical, developmental and other physiological characteristics, e.g. [11, 20, 26]. Thus, cultivation under standardized conditions should be very useful, such as a sterile *in vitro* culture [23] or as a photoautotrophic cell culture [27]. Another approach could be isolating the gene from expression libraries of *M. polymorpha* using antibodies directed against CHS from higher plants.

EXPERIMENTAL

Plant material. Marchantia polymorpha was grown in a greenhouse or in the garden of the Botanical Institute (Cologne, F.R.G.). Fresh green gametophyte material was harvested, frozen in liquid N_2 and stored at $-80°$.

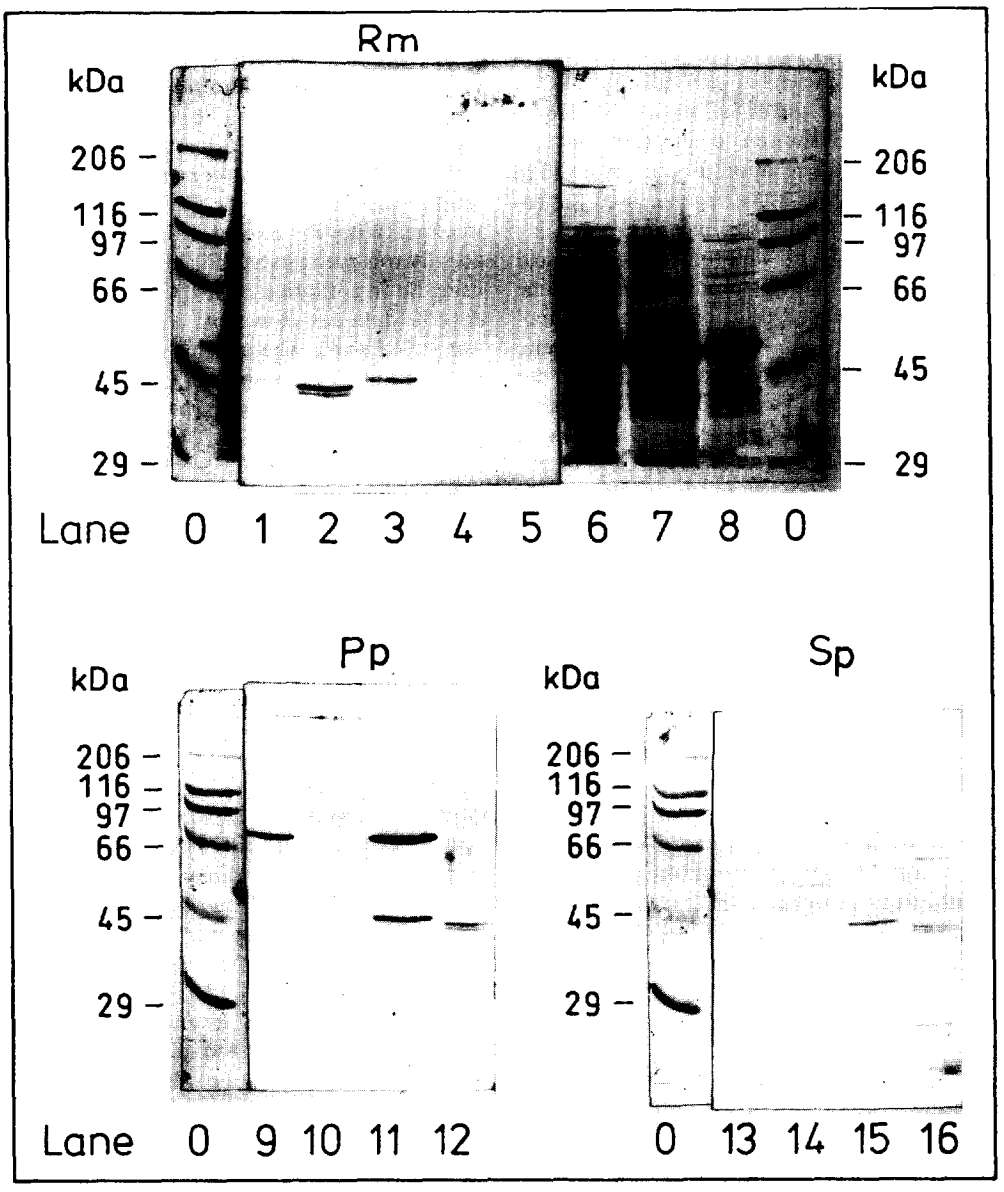

Fig. 2. SDS–PAGE and Western blot analysis of protein fractions from *M. polymorpha* and rye. Antibodies used: Rm (lanes 1–5), Pp (lanes 9–12), Sp (lanes 13–16). Lanes 6–8 stained with amido black. Immunostain of *M. polymorpha*: crude extract (8.2 μg each) = lanes 5, 9, 13; 0–50% $(NH_4)_2SO_4$ ppt. (30.8 μg each) = lanes 4, 10, 14; 50–70% $(NH_4)_2SO_4$ ppt (23.8 μg each) = lanes 3, 11, 15; sample buffer crude extract (8.2 μg) = lane 1. Immunostain of rye (control): crude extract (20.4 μg each) = 2, 12, 16. General protein stain (Amido Black) of *M. polymorpha*: 50–70% $(NH_4)_2SO_4$ ppt; 0–50% $(NH_4)_2SO_4$ ppt; crude extract = lanes 6, 7, and 8, respectively, and of M_r markers ($\times 10^{-3}$) = lane 0. μg = Amount of protein.

Chemicals. [2-^{14}C]Malonyl-CoA (2072 Mbq mmol^{-1}) was obtained from Bio Trend Chemikalien GmbH, Köln, F.R.G. Coumaroyl-CoA was synthesized according to ref. [28]. Naringenin of HPLC quality was purchased from Sigma, Heidelberg, F.R.G. Resveratrol was the kind gift of Dr R. Hain (Bayer AG, Leverkusen, F.R.G.).

Protein extraction. All steps were carried out at 0–4°. (a) Standard extraction of CHS: 1 g of frozen gametophyte thalli was ground in a mortar with liquid N_2 and extracted with 3 ml extraction buffer [0.1 M K-Pi pH 6.0, 18 mM L-cysteine, 31 mM Na ascorbate, 2% (w/v) PEG] for 40 min. After filtration through Miracloth, the homogenate was centrifuged at 30 000 *g* for 20 min. The supernatant was used for determination of enzyme activity. Protein concn was measured according to ref. [29]. (b) $(NH_4)_2SO_4$ pptn of protein including CHS: frozen plant material (15 g) was ground in a mortar with liquid N_2. The frozen powder was mixed with 45 ml extraction buffer (0.1 M K-Pi pH 8, 18 mM L- cysteine, 31 mM Na ascorbate, 1.5 g Dowex, 1.5 g PVP) and extracted for

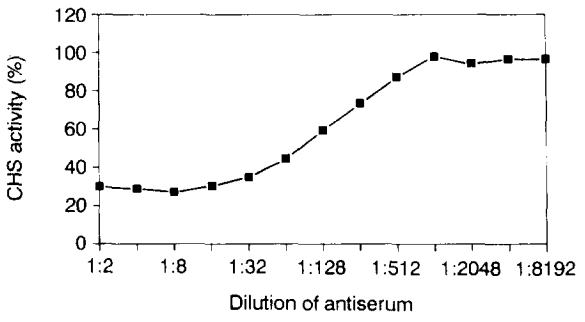

Fig. 3. Immunoprecipitation of CHS from *M. polymorpha* using the 50–70% $(NH_4)_2SO_4$ protein fraction with anti-CHS (parsley) serum.

40 min. The homogenate was filtered through Miracloth and then centrifuged at 30000 *g* for 30 min. The crude extract was pptd using 0–50 and 50–70% of $(NH_4)_2SO_4$, respectively. The $(NH_4)_2SO_4$ pellets were dissolved in 2.5 ml buffer (0.1 M K-Pi, pH 6, 18 mM L-cysteine, 31 mM Na ascorbate) and then applied to a PD 10 column (Sephadex G 25, Pharmacia). Elution was done with 3.5 ml of the same buffer. After measurement of enzyme activity and protein concn, the eluent was used for SDS–PAGE with subsequent Western blotting and for immunoprecipitation. In comparison with the crude extract at pH 8 in the 50–70% ppt CHS activity could be raised 5–6 times, protein content 3–4 times.

Chalcone synthase assay. The reaction mixture consisted of 85 μl assay buffer [250 mM K-Pi pH 8, 20 mM L-cysteine, 2% (w/v) BSA], 15 μl protein extract (15 μl crude extract of pH 6 contains 3–6 μg protein, of pH 8, 7–13 μg), 5 μl *p*-coumaroyl-CoA (208 μM) and 5 μl [2-^{14}C]malonyl-CoA (220 μM). The reaction was performed as described in ref. [11]. Combining the activity of three assays product identification was carried out via TLC in 4 dimensions on the same cellulose plate, subsequently: $CHCl_3$–HOAc (3:2:H_2O satd); C_6H_6–HOAc–H_2O (115:72:3); 30% MeOH; 15% HOAc.

Product analysis via HPLC. To synthesize enough reaction product for the HPLC experiment the 50–70% $(NH_4)_2SO_4$ ppt was used for the assay and the vol. of the assay was doubled. The reaction was stopped with MeOH after 30 min. The EtOAc layer of 20 assays were collected and evpd to dryness. Afterwards, 5 μl MeOH containing 17 nmol authentic naringenin were added. After addition of 40 μl 50% aq. MeOH, 40 μl were applied to a HPLC column (Spherisorb C8, 5 μm, 125 mm; Bischoff, Leonberg, F.R.G.). For a good separation 1% H_3PO_4 in H_2O together with organic solvents was generally used. To avoid H_3PO_4 in the eluent which drastically influences R_f values during TLC analyses, the column was first conditioned with H_2O:1% H_3PO_4 and then washed with H_2O until the eluent showed the same pH as the H_2O. Separation was performed on a linear gradient from 0–100% MeCN in H_2O over 25 min and a flow rate of 1 ml min^{-1}. The detection wavelength was 330 nm, retention time of the authentic naringenin was 17.8 min. The eluent was collected by a fraction collector with a fraction period of 30 sec (0.5 ml). One hundred microlitres of each fraction was used for scintillation counting. For subsequent TLC selected fractions (Fig. 1) were analysed, evapd with N_2 and redissolved in EtOAc containing authentic naringenin. TLC was performed on cellulose plates in 4 different solvent systems (v/v each): C_6H_6–HOAc–H_2O (115:72:3) R_f (each) of naringenin = 0.82; $CHCl_3$–HOAc (3:2:H_2O satd) R_f 0.84; EtOAc–1-PrOH–H_2O (4:1:2/upper) R_f 0.95; 30% MeOH; and on silica plates in 5 different solvent systems: C_6H_6–HOAc (17:3) R_f 0.25; C_6H_6–MeOH (9:1) R_f 0.41; EtOAc–C_6H_6 (2:1) R_f 0.72; $CHCl_3$–EtOAc–H_2O (5:4:1/lower) R_f 0.67; C_6H_6–EtOAc–MeOH–H_2O (6:4:1:3/upper) R_f 0.64.

SDS–PAGE and immunoblotting. Protein fractionation was performed on 12% SDS slab gels using the Mini Protean II Cell from Bio Rad. Crude extract of 5-day-old rye primary leaves was obtained as described elsewhere [11]. Buffers were prepared according to ref. [30]. For direct extraction of proteins in sample buffer, 100 mg of ground plant material was mixed with 200 μl of sample buffer for 1 min and afterwards heated at 95° for 4 min. M_r markers were purchased from Sigma (Marker set SDS-6H): myosin (205000), β-galactosidase (116000), phosphorylase *b* (97400), bovine albumin (66000), egg albumin (45000), carbonic anhydrase (29000). After electrophoresis, electrophoretic transfer of proteins to nitrocellulose (Pharmacia, 0.2 μm) and further processing was performed as described previously [11]. For general protein staining, sections of the nitrocellulose sheet were cut off and stained with Amido Black. For immunostaining, nitrocellulose was incubated with anti-CHS antibodies in PBS-1% BSA-0.025% Tween. The dilution of the monoclonal anti-CHS (rye) antibodies (Rm) was 1:250, for the antiserum against parsley CHS (Pp) 1:800 and for the antibodies against spinach CHS (Sp) 1:500. For the second incubation, goat-anti-rabbit (mouse) Ig conjugated to alkaline phosphatase was diluted 1:2000 and 1:1000, respectively, with PBS–1% BSA–0.025% Tween. For phosphatase staining 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) in combination with nitroblue tetrazolium (NBT) in 150 mM Tris–HCl buffer (pH 9.6) 2 mM $MgCl_2$ was added.

Immunoprecipitation. Immunoprecipitation was carried out according to ref. [31]. Equal vols of the 50–70% $(NH_4)_2SO_4$ ppt. and antibodies serially diluted with PBS/2% BSA (end vol. 40 μl) were incubated for 1 hr at room temp., followed by an incubation at 4° for 15 hr. After a centrifugation for 5 min at 8900 *g* 30 μl of the supernatant were used for the CHS assay. $(NH_4)_2SO_4$ pptd enzyme plus PBS/2% BSA served as a control.

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