Cloning and Heterologous Expression of Flavonoid Genes of Osteospermum Hybrids

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Abstract

Petals of Osteospermum exhibit white, yellow and rose to lilac colours. Chemical and biochemical investigations elucidated the basis of flower colour in Osteospermum. Besides carotenoids, which were shown to be responsible for the yellow colour, flavonoids were identified to be the main colour-giving compounds. Different amounts of derivatives of the anthocyanidin delphinidin cause the rose to lilac colour range. In addition to delphinidin, derivatives of flavonols were found to be present in the petals.

Enzyme assays elucidated the biosynthetic path leading to the formation of the 3’-, 4’-, and 5’-hydroxylated delphinidin. Because of distinct substrate specificities of the early enzymes of the flavonoid pathway, chalcone synthase, chalcone isomerase, and flavanone 3-hydroxylase, dihydrokaempferol is supposed to be the main branch point. This compound was shown to be hydroxylated in 3’- and 5’-position by flavonoid 3’, 5’-hydroxylase to dihydromyricetin which is subsequently reduced by dihydroflavonol 4-reductase to leucodelphinidin, the precursor of delphinidin.

Molecular biological investigations of two key steps allowed a confirmation of the enzymological findings. The protein of a heterologously expressed full length chalcone synthase cDNA-clone was shown to preferentially use 4-coumaroyl-CoA as a substrate leading to the formation of naringenin after isomerisation by chalcone isomerase. In contrast to this, caffeoyl-CoA as a substrate resulted in the formation of only low amounts of eriodictyol. 3’, 5’-hydroxylation, which is indispensable for the formation of delphinidin, was demonstrated with the protein of a heterologously expressed F3’5’H full-length cDNA-clone. Fragments of the other main structural flavonoid genes of Osteospermum are now available and further molecular biological investigation will be performed.

INTRODUCTION

Osteospermum originates in South Africa. Within the last 10 years it expanded to an important ornamental plant. This astonishing development is mainly due to its appealing and attractive flowers, which show white, yellow, and especially rose to lilac colours. The aim of this study was to elucidate the basis of flower colour in Osteospermum and, in particular, to understand the formation of the anthocyanidin pattern using biochemical and molecular biological methods.

In Asteraceae flowers, yellow and orange hues are mainly delivered by carotenoids (Valadon and Mummery, 1967; Goodwin, 1988) and red, blue and lilac colours by anthocyanidins (Bohm and Stuessy, 2001), the main group of colour-giving flavonoids. An important impact on expression of flower colour is accredited to other groups of flavonoids, in particular to flavones and flavonols (Brouillard and Dangles, 1993).

The determination of the hydroxylation pattern of flavonoids is mainly due to the
substrate specificity of chalcone synthase (CHS) and the presence of a flavonoid 3'-hydroxylase (F3'H) and/or flavonoid 3', 5'-hydroxylase (F3'5'H) (see Fig. 1). 4-coumaroyl-CoA and caffeoyl-CoA are possible substrates for CHS catalysing the condensation reaction with 3 units of malonyl-CoA leading to the formation of the 4'-hydroxylated naringenin (NAR) and the 3', 4'-hydroxylated eriodictyol (ERI), respectively, after isomerisation by chalcone isomerase (CHI). As observed in many cases, 4-coumaroyl-CoA is the preferred substrate (Forkmann and Heller, 1999).

F3'H and F3'5'H can hydroxylate the flavanone NAR as well as the dihydroflavonol dihydrokaempferol (DHK). The conversion of flavanones to dihydroflavanols is catalysed by flavanone 3-hydroxylase (FHT). Furthermore, the flavanones can be converted to flavones by flavone synthases (FNS) and the dihydroflavonols to flavonols by flavonol synthase (FLS) or to leucoanthocyanidins by dihydroflavonol 4-reductase (DFR). Starting from leucoanthocyanidins, the further activity of anthocyanidin synthase (ANS) and flavonol 3-O-glycosyltransferase (FGT) is necessary for the formation of anthocyanidins.

After the demonstration and extensive characterization of the activity and substrate specificity of the enzymes involved in flavonoid biosynthesis during the 1980s, the attention was drawn on cloning and expression of the respective genes in the 1990s (Davies and Schwinn, 1997; Tanaka et al., 1998; Forkmann and Heller, 1999; Winkel-Shirley, 2001).

Especially the expression of structural and regulatory genes in plants led to a deeper understanding of molecular mechanisms related to flavonoid formation and generally to metabolic processes. Nowadays, at least several clones of all main structural flavonoid genes are available. Comparison, analysis and directed modification of the nucleic acid and amino acid sequences will lead to a deeper understanding of the respective function of the proteins (Szklarz and Halpert, 1997; Johnson et al., 2001).

MATERIAL AND METHODS

Different coloured petals of Osteospermum-hybrids (Cape Daisy, Aarhus, Denmark) were examined. For enzyme assays and cloning of genes, the strong lilac cv. ‘Bamba’ was used. Plants were grown in the greenhouse under standard conditions.

Petals were extracted with MeOH and 1% MeOH/HCl and analysed spectrophotometrically. The extracts were concentrated, hydrolysed and analysed by thin layer chromatography (TLC) as described in Harborne (1984) and Martens (2000).

For enzyme assays, soluble proteins were prepared as described in Martens (2000) and the microsomal fraction as described in Diesperger et al. (1974). Protein was determined by the method of Bradford (1976). Enzyme assays were performed using [14C]-radiolabelled substrates from our lab collection. A standard enzyme assay was deduced from assays with different incubation times and protein contents and consisted in 100 mM Kpi, pH 7,5, 0,03 nmol labelled substrate, appropriate cofactors, and ca. 10-20 µg protein extract in a total volume of 100 µl. For assays performed in an extended pH range, Britton-Robinson I buffer was used. After incubation at 25°C, flavonoids were extracted with EtOAc and separated by TLC. Radioactivity was quantified with the Bio-Imaging Analyser (Fuji Bas 1000).

Total RNA was prepared from petals of the strong lilac cultivar ‘Bamba’ with the RNeasy Kit (Quiagen). For RT-PCR, degenerated primers complementary to conserved regions of the desired genes were used. For genes of special interest (CHS, DFR, F3'5'H, FLS, FGT) the obtained fragments were completed by RACE-PCR-techniques as described in Eder (2001). The sequences were analysed with standard molecular biological programs. The full-length cDNA-clones were cloned into the expression vector pYES2.1/V5-His-TOPO® (Invitrogen) and expressed heterologously in baker’s yeast (S. cerevisiae) as described in Martens (2000). Enzyme assays were performed as described above.
RESULTS AND DISCUSSION

Chemical Basis of Flower Colour

Methanolic extracts of different coloured petals showed a typical absorption spectra for carotenoids (Fig. 2). As expected, the absorption maximum of yellow petals was significantly higher than that of lilac or white coloured petals, which show only a pale yellow coloration on the lower part of their petals. MeOH/HCl extracts of lilac coloured petals exhibited the absorption optima around 540 nm indicating the presence of derivatives of delphinidin (Dp) (Fig. 2). There was a clear difference in absorption between strong and weak lilac petals.

The separation of hydrolized extracts on thin layer chromatography confirmed that Dp is the basic colour-giving compound in coloured petals. Derivatives of cyanidin (Cy) were found only in smaller amounts. The flavonols kaempferol (Km), quercetin, and myricetin were detected in hydrolysed extracts, too. Flavonols are colourless pigments but contribute to the expression of flower colour as a co-pigment together with anthocyanins.

Enzymological Investigations

Using both 4-coumaroyl-CoA and caffeoyl-CoA in one assay mixture as starter molecules together with [1-14C]-malonyl-CoA and with crude enzyme extract, the predominant formation of NAR was observed (Fig. 3). Therefore, 4-coumaroyl-CoA seems to be the natural substrate for CHS under physiological conditions. In contrast to this, caffeoyl-CoA probably is not used as a substrate in vivo because the synthesis of the 3’, 4’-hydroxylated ERI occurred only in small amounts at low pH values.

Since NAR exhibits only one hydroxy group at the 4’-position at the B-ring, the activity of a F3’5’H is necessary for the formation of 3’, 4’, 5’-hydroxylated flavonoids like the derivatives of Dp. In fact, incubation of the microsomal fraction with NADPH and NAR or DHK as substrates led to the formation of the 3’, 4’, 5’-hydroxylated products, pentahydroxyflavanone (PHF) or dihydromyricetin (DHM), respectively. When both substrates were offered in the same assay, NAR was the preferred substrate.

Despite this finding, hydroxylation is supposed to occur on the level of dihydroflavonols because NAR was shown to be the preferred substrate for FHT. In relation to NAR, ERI was converted to dihydroquercetin (DHQ) only to 30% by FHT under standard conditions and PHF did not serve as a substrate at all.

Finally, DFR activity was detected catalysing the reduction of DHM to leucodelphinidin, the precursor of Dp.

The decisive role of F3’5’H-activity concerning anthocyanin formation is illustrated by a simple experiment consisting in the treatment of buds with the cytochrome P450 inhibitor tetcyclacis. Treated petals exhibited a drastic decrease in synthesis of Dp derivatives, an increase in Km derivatives (see also Fig. 1) and remained almost acyanic.

Molecularbiological Approaches

In order to confirm and widen the knowledge about flavonoid biosynthesis in Osteospermum, the isolation of flavonoid genes was started. By the use of degenerated primers, fragments of the main structural genes were obtained. These fragments exhibited high homologies to already cloned flavonoid genes especially those of other Asteraceaen species (Tab. 1, 2 and 3). For genes of special interest (CHS, DFR, F3’5’H, FLS, FGT) the obtained fragments were completed by RACE-PCR-techniques and heterologously expressed in yeast. In accordance to the enzymological data, protein of the expressed cDNA-clone of CHS preferentially used 4-coumaroyl-CoA as a substrate, whereas caffeoyl-CoA was only used to a low extend.

The CHS sequences of Asteraceaen species show high homologies around 90% in amino acid sequence and more than 80 % in nucleotid sequence (Tab. 2). But there are homologies around 80 % and 70 %, even to the CHS sequence of the monocotyledonous plant Triticum aestivum, demonstrating the conserved structure of CHS.

The demonstration of the function of the F3’5’H-clone was of particular interest.
This clone has a F3‘H-like sequence as indicated by alignment and homology calculation (Tab. 3). For example, the homology to the amino acid sequence of F3‘H of Petunia hybrida amounts to 67% and only to 48% to the sequence of F3‘5‘H of Petunia. Therefore, 3‘-hydroxylation ought to be expected. But undoubted, protein of the expressed cDNA-clone showed strong F3‘5‘H-activity instead of F3‘H-activity (Fig. 4). DHK was predominantly 3‘, 5‘-hydroxylated to DHM whereas 3‘-hydroxylation to DHQ occurred only to a low extent. As observed with the microsomal fraction prepared from petals, NAR is the preferred substrate in comparison to DHK.

The phenomenon described has been already observed with Callistephus chinensis (Eder, 2001). One clone isolated from flowers of Ç. callistephus showed high homlogies to F3‘H-sequences, too, but heterologous expression exhibited 3‘, 5‘-hydroxylation activity although 5‘-hydroxylation was weak compared to those observed with Osteospermum.

Conclusions

The combination of chemical, biochemical, and molecular biological methods led to a basic understanding of flavonoid formation in Osteospermum flowers. Because sequence information about all main structural genes is available now, further studies like Northern or Southern blotting can be performed. Due to the high homology of flavonoid genes in Asteracean species, comparative analysis of these sequences in conjunction with the knowledge about the biochemical characteristics of the proteins will reveal valuable information about the amino acids, which play a decisive role in respect to the function and substrate specificity of the proteins. In this respect, construction of chimeric genes and site-directed mutagenesis are powerful tools to widen the knowledge about the molecular reality.

ACKNOWLEDGEMENTS

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Literature Cited


Martens, S. 2000. Genetische, biochemische und molekularbiologische Untersuchungen


### Tables

Table 1. Fragments of flavonoid genes isolated from *Osteospermum*. The homology values are calculated by BlastX program 2.2.6 (NCBI).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Length of clone</th>
<th>% Homology to other species in nucleotid/amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>chalcone synthase (CHS)</td>
<td>398 AS, full length</td>
<td>see table 2</td>
</tr>
<tr>
<td>flavanone 3-hydroxylase (FHT)</td>
<td>670 bp-fragment (including 3’-end)</td>
<td><em>Callistephus chinensis</em> 88/95 <em>Chrysanthemum x morifolium</em> 84/90 <em>Matthiola incana</em> 77/85</td>
</tr>
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<td>dihydroflavonol 4-reductase (DFR)</td>
<td>365 AS, full length</td>
<td><em>Gerbera x hybrida</em> 85/91 <em>Callistephus chinensis</em> 84/90 <em>Rhododendrom simii</em> 82/91</td>
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<tr>
<td>anthocyanidin synthase (ANS)</td>
<td>831 bp-fragment (including 3’-end)</td>
<td><em>Callistephus chinensis</em> 82/90 <em>Malus x domestica</em> 74/87 <em>Ipomoea Nil</em> 75/87</td>
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<tr>
<td>flavonoid 3’,5’-hydroxylase (F3’5’H)</td>
<td>508 AS, full length</td>
<td><em>Callistephus chinensis</em> 76/87 <em>Arabidopsis thaliana (F3’H)</em> 63/79 <em>Petunia x hybrida (F3’H)</em> 67/82</td>
</tr>
<tr>
<td>flavonol synthase (FLS)</td>
<td>335 AS, full length</td>
<td><em>Petroselinum crispum</em> 74/86 <em>Eustoma grandiflora</em> 73/86 <em>Petunia x hybrida</em> 72/84</td>
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<tr>
<td>flavonoid 5-O-glycosyltransferase (FGT)</td>
<td>348 AS, full length</td>
<td><em>Petunia x hybrida</em> 59/76 <em>Verbena x hybrida</em> 59/73 <em>Perilla frutescens</em> 57/73</td>
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</table>
Table 2. Homology matrix in % between different CHSs calculated by the multiple alignment program CLUSTAL W. On the right upper part homology between amino acid sequences, on the left lower part homology between nucleic acid sequences.

<table>
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<th>Call</th>
<th>Gerb</th>
<th>Vitis</th>
<th>Petro</th>
<th>Bet</th>
<th>Dauc</th>
<th>Ruta</th>
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<td>72</td>
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<td>71</td>
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Table 3. Homologies in % between amino acid sequences of different F3'Hs and F3'5'Hs calculated by the multiple alignment program CLUSTAL W.

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<th></th>
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<th>Tor-3’</th>
<th>Pet-3’5’</th>
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</table>

3’ = F3’H, 3’5’ = F3’5’H, Osteo = *Osteospermum hybrida*, CC = *Callistephus chinensis*, Pet = *Petunia hybrida*, Tor = *Torenia hybrida*
Fig. 1. General biosynthetic pathway of flavonoids.
Fig. 2. Spectrophotometric measurement of MeOH- (left) and 1% MeOH/HCl-extracts (right) of different coloured petals.

Fig. 3. CHS-assays containing crude protein extract of petals, [14C]-malonyl-CoA, and 4-coumaroyl-CoA as well as caffeoyl-CoA as substrates.

Fig. 4. Heterologous expression of F3’5’H. Besides F3’5’H-protein the enzyme assays contained NADPH and [14C]-DHK.