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Betaxanthin formation and free amino acids in hairy roots of *Beta vulgaris* var. *lutea* depending on nutrient medium and glutamate or glutamine feeding

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Abstract

Feeding of amino acids to hairy roots of the yellow beet (*Beta vulgaris* var. *lutea*) usually results in the formation of the respective betaxanthins. One exception is (*S*)-glutamate whose feeding leads to an increase in the betaxanthin vulgaxanthin I (glutamine as amino-acid moiety) instead of vulgaxanthin II (glutamate as amino-acid moiety). To elucidate this phenomenon, hairy roots were cultivated in modified standard medium and (*S*)-glutamate was fed. Under most nutrient conditions tested, glutamine and vulgaxanthin I in the tissue dominated over glutamate and vulgaxanthin II. Glutamate, opposed to glutamine, was readily metabolized so that its concentration was lower than that of glutamine. Maximum concentrations of glutamate were reached when the activity of glutamine synthetase was low. Even then, however, vulgaxanthin II stayed on a low level. In contrast, the level of vulgaxanthin I increased with increasing concentrations of glutamine in the tissue. Also 4-aminobutyric acid (GABA) was a major amino acid in the hairy roots. Its concentration reached maximum levels when (*S*)-glutamate, a GABA precursor, was fed, or when sucrose, the C source of the roots, was replaced by glucose. The respective GABA–betaxanthin, however, was hardly detectable. When both (*S*)-glutamate and glucose were supplied, the GABA concentration exceeded that of all other amino acids. Only then the GABA–betaxanthin could be characterized in small amounts. Interestingly, the level of the main betaxanthin, miraxanthin V, consisting of betalamic acid and dopamine, was most markedly reduced by a replacement of sucrose with glucose.

We conclude that the reaction of betalamic acid with glutamate and GABA was considerably lower than with glutamine and dopamine, irrespective of the concentration of the amino acid in the tissue. Possible reasons will be discussed, also with respect to the occurrence of species-specific patterns of betaxanthins.

Keywords: *Beta vulgaris* L. var. *lutea*; Chenopodiaceae; Yellow beet; Hairy roots; Nutrient media; (*S*)-Glutamate feeding; Glutamine synthetase; Amino acids; Betaxanthins

1. Introduction

Betaxanthins and betacyanins are yellow and red-violet, nitrogen-containing pigments, named betalains. They are conjugates of betalamic acid with amino acids or amines and *cyclo*-DOPA or 2-descarboxy *cyclo*-DOPA, respectively (Steglich and Strack, 1990; Kobayashi et al., 2001). Betalains are characteristic for nearly all families of the order Caryophyllales and they

Abbreviations: DOPA, 3,4-dihydroxyphenylalanine; FW, fresh weight; GABA, 4-aminobutyric acid; GDH, glutamate dehydrogenase; Glc, glucose; Gln, glutamine; Glu, glutamate; GOGAT, glutamate oxoglutarate amino transferase; GS, glutamine synthetase.

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also occur in some higher fungi. Furthermore, it is of chemotaxonomic interest that they co-exist with different flavonoids but not with anthocyanins (Steglich and Strack, 1990). Due to their distribution in the plant kingdom, betalains are components of various foodstuffs, e.g., roots of red and yellow beet (Kujala et al., 2000; Stintzing et al., 2002), leaves of foliage beet and Indian spinach (Zakharova and Petrova, 1997; Gläßgen et al., 1993), fruits of *Opuntia ficus-indica* (Stintzing et al., 2001) and tubers of *Ullucus tuberosus* (Böhm, unpubl. results). Additionally, betacyanins are increasingly used as food dyes (Stintzing et al., 2000, 2001; Cai and Corke, 2000). With respect to a remarkable intake of betalains, it appears important that these secondary substances exhibit antioxidant activities (Escribano et al., 1998; Kanner et al., 2001; Tesoriere et al., 2003). They could, therefore, play a role in the prevention of chronic diseases caused by reactive oxygen species in humans (Halliwell, 1997).

Although many betacyanins and betaxanthins are structurally known since 1960, their biosynthetic pathways were elucidated in detail during the past 10 years (Strack et al., 2003). A result is the conclusion that betalamic acid condenses with its partner molecule in a non-enzymatic reaction. This finding is essentially based on experiments in which feeding of hairy roots or seedlings of beets with different (*S*)- and even (*R*)-amino acids resulted in the formation of the respective betaxanthins (Hempel and Böhm, 1997; Schliemann et al., 1999). These experiments, however, showed throughout one exception: Feeding of (*S*)-glutamate did not lead to the formation of vulgaxanthin II (Fig. 1), the respective condensation product, but instead to an increase of vulgaxanthin I (Fig. 1) which contains glutamine as amino-acid moiety. Since the attempt to obtain a higher amount of vulgaxanthin II by inhibition of GS failed (Hempel and Böhm, 1997), further investigations were performed in the present study. Hairy roots of yellow beet were cultivated in modified B50 nutrient medium and (*S*)-glutamate was fed with the aim to accumulate this amino acid in the plant tissue. Amino-acid analyses were paralleled by qualitative and quantitative investigations of the betaxanthins, especially of vulgaxanthin II. From the comparison of both metabolic areas, we intended to answer the question described before and also to contribute to an understanding of the problem how species-specific patterns of betaxanthins can occur (Steglich and Strack, 1990) despite the absence of an enzymatic regulation of the decisive biosynthetic step, the condensation.

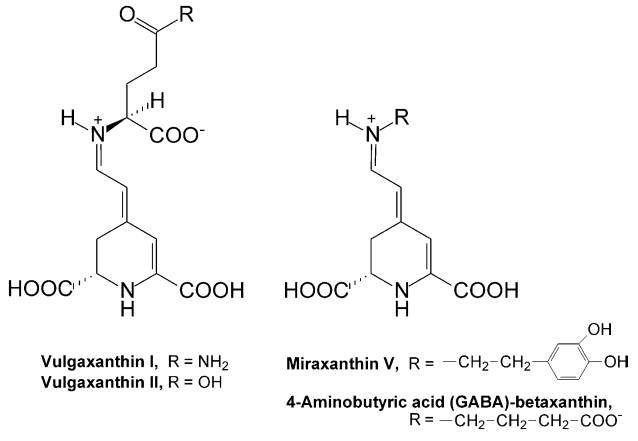

Fig. 1. Structures of betaxanthins occurring in the hairy roots.

2. Results and discussion

2.1. Effect of nutrient media and feeding of glutamate/glutamine on the concentration of total free amino acids

Growth in the standard medium (B50, after Gamborg et al., 1968) resulted in a relatively low concentration of total free amino acids in the hairy roots (Fig. 2(a)). Interestingly, a reduction of the nitrate concentration in the nutrient medium to one-tenth (B50/10) of the standard medium did not further decrease the amino-acid concentration. This indicates that the pool of free amino acids was kept constant over a wide range of external nitrate concentrations. Changes in the nitrate supply became more obvious in the protein concentration (Fig. 2(b)) which was approximately 50% lower after growth in the low-nitrate medium (B50/10) compared to the standard medium (B50). When sucrose was replaced by glucose (B50 Glc and B50/10 Glc), GS activity (Fig. 2(c)) and protein concentration (Fig. 2(b)) declined, most pronouncedly in the low-nitrate medium (B50/10 Glc). This confirms Morcuende et al. (1998) who reported that nitrogen assimilation in leaves of tobacco was stimulated more by sucrose than by glucose. In the hairy roots, the replacement of sucrose by glucose in the medium resulted in maximum levels of free amino acids under high-nitrate conditions (B50 Glc) but not under low-nitrate conditions (B50/10 Glc).

When (*S*)-glutamate (+Glu) or (*S*)-glutamine (+Gln) was added to the nutrient media the concentration of total free amino acids increased by at least a factor of 2 (Fig. 2(a)). This clearly shows that the amino acids were taken up by the roots. GS activity (Fig. 2(c)) and protein content (Fig. 2(b)) declined, most likely as a result of feedback inhibition of GS by the supplied amino acids. When Hempel and Böhm (1997) inhibited GS with methionine sulfoximine vulgaxanthin I formation was inhibited but no effect on vulgaxanthin II was observed. In the present experiments, reduced activities of GS were accompanied by slightly increased vulgaxanthin II peaks (compare Fig. 2(c) and Table 2). We assume that this increase is a consequence of the increased glutamate concentrations (Table 1) which resulted from (*S*)-glutamate feeding and which caused the decline in GS ac-

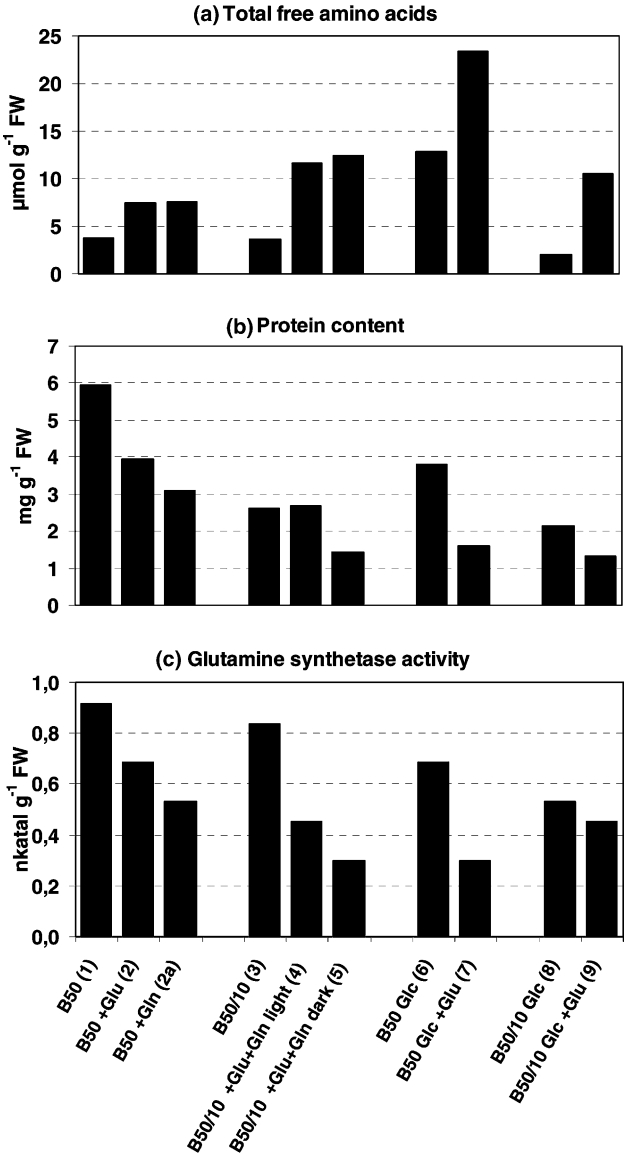

Fig. 2. (a) Concentration of total free amino acids, (b) protein and (c) activity of glutamine synthetase in the hairy roots under the respective nutrient conditions (medium 1–9).

tivity. When GS was inhibited by methionine sulfoximine probably much less glutamate accumulated.

Under all nutritive conditions, GS activity (Fig. 2(c)) showed the same trend like the protein content (Fig. 2(b)). In fact, both parameters correlated quite well with a Pearson correlation coefficient of $R = 0.813$. This demonstrates the importance of GS in nitrogen assimilation in hairy roots of yellow beet. GDH probably played only a minor, if any, role under the tested conditions because its activity is inhibited by sugars (Oaks et al., 1980; Sahulka and Lisá, 1980; Tassi et al., 1984). A cytosolic and a plastidic isoform of GS are active in the hairy roots (Mäck, 1998). Feeding in darkness resulted in a reduction of the activity of the predominant cytosolic isoform (B50/10 + Glu + Gln dark, Fig. 2(c); Mäck, 1998).

To summarize, nitrate plus sucrose (and light) resulted in high GS activity and protein synthesis so that only a small pool of free amino acids remained in the roots. This pool was increased by severalfold when (*S*)-glutamate or (*S*)-glutamine was co-supplied with nitrate and sucrose, and even more when sucrose was replaced by glucose. The highest concentration of free amino acids was achieved by a combination of glucose and (*S*)-glutamate; their concentration under this nutrient condition (B50 Glc + Glu) was 6-fold higher than in the standard medium (B50).

2.2. Effect of the nutrient media and feeding of glutamate/glutamine on the pattern of total free amino acids

After growth in the standard medium (B50) the ratio of glutamate:glutamine was 2.5:1 (medium 1, Table 1). This corresponds closely to the theoretical value of the GS/GOGAT reaction. In the low-nitrate media (medium 3 and 8, Table 1), the ratio was even higher. Under all other nutrient conditions tested, however, glutamate was present in the pool of free amino acids at lower concentrations than glutamine (Table 1). This is similar to conditions in the storage root of sugar beet, where a ratio of glutamate:glutamine of 1:7 was reported for plants from field trials (Schiweck et al., 1994) and of up to 1:11 when supplied hydroponically with NH_4^+ (Mäck, 1988). It is assumed that glutamate is a better amino donor for amino acid synthesis than glutamine (Heldt, 1997) so that in actively growing organs its concentration may be lower than that of glutamine. Our experiments confirm this assumption. When (*S*)-glutamate was supplied to the hairy roots, the concentration of not only glutamate but also of other amino acids increased (compare medium 1 and 2, Table 1). This response was generally observed whenever (*S*)-glutamate, either alone or in combination with (*S*)-glutamine, was fed. Glutamine supply, on the other hand, increased only its own concentration (compare medium 1 and 3, Table 1) indicating that glutamine was taken up but not readily metabolized.

Besides glutamate and glutamine, also GABA was one of the major amino acids in the hairy roots. In the storage root of sugar beet it is the 2nd to 3rd most abundant amino acid and comprises 10% of all free amino acids (Schiweck et al., 1994). It was the predominant amino acid in the hairy roots when sucrose was replaced by glucose in the high-nitrate standard medium (medium 6, Table 1) and reached its absolute highest level when (*S*)-glutamate was supplied (medium 7, Table 1). The high concentration of total free amino acids as described in the foregoing section was a consequence of these high-GABA concentrations. The sugar exchange was, however, without effect on GABA under low-nitrate conditions (compare medium 3 and 8, Table 1). GABA is synthesized from glutamate

Table 1
Concentration of free amino acids (μmol g^{-1} FW) in the hairy roots under the respective nutrient condition (medium 1–9)

	(1) B50	(2) B50 + Glu	(2a) B50 + Gln	(3) B50/10	(4) B50/10 + Glu + Gln	(5) Like (4), in darkness	(6) B50 Glc	(7) B50 Glc + Glu	(8) B50/10 Glc	(9) B50/10 Glc + Glu
Ala	0.31	0.68	0.18	0.11	0.57	2.06	0.04	1.94	0.03	0.28
Arg	0.17	0.55	0.14	0.29	0.51	0.76	1.52	0.63	0.32	0.11
Asn	0.084	0.12	0.21	0.04	0.14	0.26	0.24	0.40	n.d.	0.11
Asp	0.14	0.51	0.16	0.11	0.32	0.64	0.62	0.66	0.14	0.55
Cys	n.d.	n.d.	n.d.	0.02	n.d.	n.d.	0.04	0.13	n.d.	n.d.
GABA	0.37	1.05	0.29	0.21	0.82	0.56	2.13	6.52	0.18	0.88
Gln	0.20	1.02	4.69	0.04	4.36	2.51	0.95	3.16	0.04	3.69
Glu	0.51	0.96	0.48	0.14	1.35	2.30	0.77	1.18	0.19	2.33
Gly	n.d.	n.d.	n.d.	0.13	0.31	0.24	0.35	0.49	0.10	n.d.
His	0.07	0.12	0.11	0.05	0.19	0.27	0.11	0.12	0.08	0.06
Ile	0.083	0.06	0.11	0.40	0.09	0.16	0.51	0.33	0.04	0.12
Leu	0.20	0.38	0.19	0.32	0.31	0.40	1.12	1.15	0.06	0.31
Lys	0.20	0.27	0.12	0.20	0.18	0.62	0.93	0.78	0.06	0.11
Met	n.d.	n.d.	n.d.	0.05	n.d.	0.03	0.27	0.30	0.04	0.10
Phe	0.11	0.08	0.11	0.07	0.10	0.13	0.37	0.39	0.03	0.10
Pro	0.54	0.71	0.26	0.22	0.66	0.36	0.70	2.27	0.34	0.62
Ser	0.17	0.22	0.14	0.42	0.54	0.52	0.80	0.84	0.07	0.23
Thr	0.47	0.43	0.18	0.47	0.62	0.34	0.61	1.33	0.24	0.56
Tyr	n.d.	0.04	n.d.	0.03	0.10	n.d.	0.03	0.06	0.02	0.06
Val	0.17	0.15	0.18	0.25	0.28	0.26	0.71	0.74	0.06	0.33
Total	3.80	7.37	7.55	3.59	11.46	12.44	12.82	23.40	2.03	10.54

n.d., not detectable.

(Morot-Gaudry et al., 2001), and this has reached minimum levels under low-nitrate conditions. It must thus be assumed that glucose stimulated GABA synthesis only when glutamate was present in sufficient concentrations.

To summarize, it was possible to increase the glutamate concentration in the pool of free amino acids by modifying the standard medium. The highest glutamate concentrations were achieved in the low-nitrate medium supplied with (*S*)-glutamate either in darkness (B50/10 + Glu + Gln dark, medium 5), or in the light when sucrose was replaced by glucose (B50/10 Glc + Glu, medium 9). Under these nutrient conditions, GS had reached low activities (Fig. 2(c)) and, consequently, glutamate accumulated. For similar reasons, high glutamate concentrations should be expected also under the B50 Glc + Glu condition (medium 7), but, as outlined above, here glutamate was consumed for GABA synthesis.

2.3. Betaxanthin formation: time course of feeding of glutamate

Earlier feeding experiments with (*S*)-glutamate were performed for a relatively long time (3 days; Hempel and Böhm, 1997). It cannot be excluded that during this time vulgaxanthin II was first formed and then turned into vulgaxanthin I under the influence of an increasing amount of glutamine produced by GS. The exchange of the amino-acid moiety in betaxanthins is known from semisynthetic work (Rink and Böhm, 1991; Trezzini and Zryd, 1991), although under alkaline conditions which do not occur in whole plants. If this process would take place in the hairy roots, the discrepancy outlined in Section 1 would in fact not exist. We therefore monitored the levels of vulgaxanthin I and vulgaxanthin II for 32 h after feeding of (*S*)-glutamate to hairy roots which were cultivated in B50/10 medium where they were free from vulgaxanthin I (see Section 2.4). As presented in Fig. 3, the increase of vulgaxanthin I was not preceded by an accumulation of vulgaxanthin II. The latter stayed rather constant whereas vulgaxanthin I

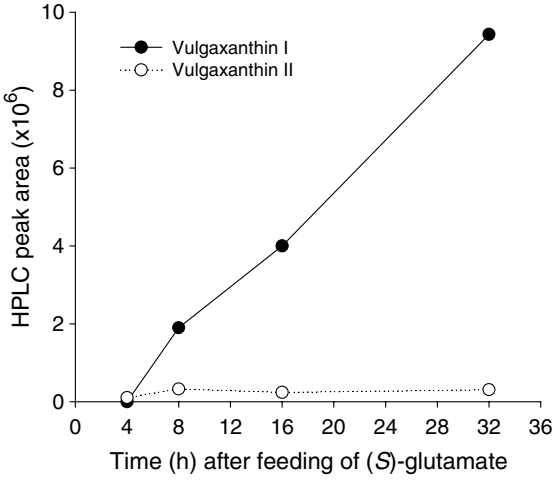

Fig. 3. Betaxanthin concentrations in the hairy roots after feeding of (*S*)-glutamate. Vulgaxanthin I was not detectable in controls (without glutamate supply), vulgaxanthin II showed control values of 0.33×10^6 and 0.27×10^6 at 4 and 32 h, respectively.

was directly formed after feeding of glutamate. These findings substantiate our view of the discussed problem.

2.4. Betaxanthin formation: effect of the nutrient media

The betaxanthin pattern after growth of hairy roots in the standard medium (B50) confirmed previous results (Hempel and Böhm, 1997; Schliemann et al., 1999). During cultivation in the last years the quantity of the main compound, miraxanthin V, the betalamic conjugate of dopamine (Fig. 1), had enlarged and that of vulgaxanthin I had remarkably decreased so that presently a relationship resulted as indicated in line 1 of Table 2. The quantitative superiority of vulgaxanthin I over vulgaxanthin II as expected from earlier work was also found which surprisingly was accompanied by an inverse ratio of free glutamine and glutamate (medium 1, Tables 1 and 2).

When the nitrate content of the nutrient medium was reduced to one-tenth of the standard (B50/10), the hairy roots formed practically no vulgaxanthin I (medium 3, Table 2). The level of vulgaxanthin II, however, was slightly higher than under standard conditions although free glutamate had reached a minimum in the low-nitrate supply (medium 3, Table 1). Also miraxanthin V appeared affected in the B50/10 medium (medium 3, Table 2). Nitrate limitation as reason for the absence of vulgaxanthin I was confirmed by transferring hairy roots from the B50/10 to the B50 medium. After three subcultures a vulgaxanthin I peak appeared with an area of 3.92×10^6.

The replacement of sucrose by glucose (B50 Glc) had the most marked effect on miraxanthin V whose content was lowered considerably (medium 6, Table 2). Also vulgaxanthin II was reduced, despite an increased concentration of glutamate and all other amino acids compared to standard conditions (medium 1 and 6, Table 1). GABA reached its second highest concentration and was the most abundant of all amino acids in this medium. A correspondingly large peak of GABA–betaxanthin (Fig. 1) which was recently described for the first time in yellow beets (Stintzing et al., 2002) was, however, not observed. Only a fairly small peak appeared (medium 6, Table 2).

When nitrate was reduced to one-tenth in the glucose medium (B50/10 Glc), the amount of miraxanthin V was further decreased and reached its minimum level (medium 8, Table 2). Vulgaxanthin I was absent, as expected from the results in the B50/10 medium. The vulgaxanthin II peak was similar to that in the B50/10 medium, despite a very low glutamate concentration (medium 8, Tables 1 and 2).

2.5. Betaxanthin formation: effect of feeding of glutamate and glutamine

Feeding of (*S*)-glutamate (+Glu) resulted in a marked increase or the appearance of vulgaxanthin I compared with the controls. The increase was even more pronounced when (*S*)-glutamine (+Gln) was co-supplied (medium 2, 4, 5, 7, 9, Table 2). Also increased vulgaxanthin II values were detectable after glutamate feeding, but they did not essentially exceed the control level (36% on average). In contrast, the addition of glutamate to hairy root cultures lowered the miraxanthin V amounts by approximately 10% on average (medium 2,4,5,7,9, Table 2). This decrease was probably due to the competition of an increased number of free amino acid molecules with dopamine for betalamic acid. Corresponding results were reported by Schliemann et al. (1999) who fed various amino acids also to hairy roots of yellow beet. In our experiments, the miraxanthin V decrease after glutamate feeding was more pronounced when glucose was supplied instead of sucrose. Glucose probably caused a shortage of betalamic acid. This would also explain the low concentration of miraxanthin V under the influence of the glucose media B50 Glc and B50/10 Glc (Table 2). No free betalamic acid was

Table 2
Peak areas ($\times 10^6$) of relevant betaxanthins from HPLC profiles

Nutrient medium	No.	Vulgaxanthin I $\lambda_{max} = 472$ nm	Vulgaxanthin II $\lambda_{max} = 470$ nm	GABA–betaxanthin $\lambda_{max} = 460$ nm	Miraxanthin V $\lambda_{max} = 464$ nm
B50	1	1.10	0.31	n.d.[a]	120.79[b]
B50 + Glu	2	6.06	0.56	n.d.[a]	106.46[b]
B50/10	3	0.07	0.37	n.d.[a]	102.20[b]
B50/10 + Glu + Gln	4	24.15	0.45	n.d.[a]	95.06[b]
B50/10 + Glu + Gln darkness	5	19.40	0.41	n.d.[a]	94.89[b]
B50 Glc	6	1.16	0.21	0.09	62.67
B50 Glc + Glu	7	1.74	0.23	0.34	45.95
B50/10 Glc	8	n.d.[a]	0.42	n.d.[a]	36.36
B50/10 Glc + Glu	9	2.34	0.59	n.d.[a]	30.30

[a] Not detectable.
[b] Cutoff at the 20-μV line.

detected in hairy roots grown in these media (data not shown).

Feeding of (*S*)-glutamate when glucose was replaced by sucrose (B50 Glc + Glu) was most effective on the GABA–betaxanthin (medium 7, Table 2). GABA and the respective betaxanthin increased 3-fold compared to the control medium (medium 6 and 7, Tables 1 and 2).

To focus the discussion: Even at the highest glutamate concentration of 2.3 μmol g^{-1} FW (medium 5 and 9, Table 1) vulgaxanthin II never reached peak areas which other betaxanthins usually would show after the feeding of the respective amino acids (Hempel and Böhm, 1997). The peak areas of vulgaxanthin II always stayed in the small range of 0.21×10^6–0.59×10^6 despite a large range of glutamate from 0.14 to 2.3 μmol g^{-1} FW. One reason for this finding is probably that glutamate must compete for betalamic acid with higher concentrations of glutamine (>4 μmol g^{-1} FW; medium 4, Table 1) and especially of dopamine which was found in a concentration of 15 μmol g^{-1} FW in hairy roots of yellow beet cultivated in B50 medium (Schliemann et al., 1999). Furthermore, a limited reactivity between glutamate and betalamic acid seems likely. This assumption was deduced from a comparison of the amounts of vulgaxanthin II and vulgaxanthin I under conditions of similar glutamate and glutamine concentrations (medium 2, 5, and 6, Tables 1 and 2). A limited reactivity was also indicated for GABA which reached the highest level of all amino acids (medium 7, Table 1), but was accompanied by only a relatively small peak of its betaxanthin (medium 7, Table 2). On the other hand, dopamine and glutamine showed a high reactivity with betalamic acid. However, neither glutamine and dopamine nor glutamate and GABA are identical in their chemical character. Therefore, it seems questionable that the condensation leading to betaxanthins depends primarily on the chemical properties of the amino acids or amines. An intracellular compartmentation of the reaction partners and the pH of the biosynthetically active cells should rather be assumed as the reason for differences in the formation of individual betaxanthins. This would also explain the observed species-specific patterns of betaxanthins. The importance of the intracellular localization of amino acids was already discussed by Schliemann et al. (1999). They fed betalamic acid to broad bean which under natural conditions does not synthesize betalains. The induced main betaxanthin was dopaxanthin and, although DOPA and asparagine were present in similar concentrations, no asparagine–betaxanthin could be identified.

3. Conclusion

The relatively low or even absent increase of vulgaxanthin II after the feeding of (*S*)-glutamate to hairy roots of yellow beet is not contradictory to the assumption of a spontaneous condensation between betalamic acid and its partners in the course of betaxanthin biosynthesis. The reasons for this statement are our following findings: 1. Although the feeding of glutamate led to an increased internal concentration of this amino acid it accumulated only in few cases. This was probably a consequence of the high metabolisation of glutamate in various pathways. 2. Only slightly increased peak areas of vulgaxanthin II and also of GABA–betaxanthin despite the accumulation of the respective free amino acids indicate that the absolute concentration of an amino acid, even if it dominates in the tissue, is not the only factor influencing its reaction with betalamic acid. Another factor seems to be the suitability of a certain amino acid as reaction partner. It is obviously low for glutamate and GABA in our experimental system. Conditions that determine this suitability are not yet known. The intracellular situation seems more important than the chemical character of the amino acids. The former might also explain the species-specific patterns of betaxanthins.

4. Experimental

4.1. Plant material and cultivation

4.1.1. Plant material

In the present investigation, line 5D of a hairy root culture from yellow beet (*Beta vulgaris* L. var. *lutea*) was used, the initiation and maintenance of which were described by Hempel and Böhm (1997). Line 5D was cultivated not only in B50 medium, a medium after Gamborg et al. (1968) without phytohormones, but also in the following modifications of B50: B50/10 = reduction of nitrogen to one tenth (= 0.3 g l^{-1} KNO_3); B50 Glc = equimolar replacement of sucrose by glucose; B50/10 Glc = reduction of nitrogen plus replacement of sucrose by glucose. Growth with reduced nitrogen resulted in slightly softer roots but growth rates were not affected. Glucose instead of sucrose, however, changed the morphology from a branched planar into a compact spherical structure which grew significantly slower. Segments of these balls were thus subcultivated only every 18 days compared to every 7 days when grown with sucrose.

4.1.2. Feeding of glutamate and glutamine

The amino acids (*S*)-glutamate and (*S*)-glutamine were supplied to the hairy roots after 3 subcultures in the nutrient media without amino acids. The plant material for a feeding experiment originated from the same culture flask like the controls. Glutamate or glutamine were sterile-filtrated into the nutrient media at a final concentration of 3 mM. Each feeding started 2 days before the normal subculture was finished, i.e., at the 5th day in the media

with sucrose and at the 16th day in the media with glucose. After a feeding period of ca. 36 h the plant material was harvested. Cultivation was performed in a 14:10 h light (white fluorescent tubes, 3000 cd sr m^{-2}):dark regime at 26 ± 1 °C. To investigate the effect of constant darkness, some cultures were wrapped in aluminium foil immediately after the amino-acid supplement.

An exception was the kinetics which started, like in previous experiments (Hempel and Böhm, 1997), at the 4th day of the subculture and which consisted of the feeding steps as shown in Fig. 3. Furthermore, it was performed under continuous light to provide constant conditions for betaxanthin biosynthesis throughout the entire experiment.

4.1.3. Harvest

The hairy roots were briefly washed with distilled water, gently blotted dry with filter paper and weighed. Plant material with similar weight from two replicates was halved and the halves were combined into two mixed samples. One of these was shock-frozen with liquid nitrogen and stored at −80 °C for the physiological analyses. The second was frozen at −20 °C, freeze-dried and then stored at −20 °C for the analysis of betaxanthins. In the kinetics each plant sample originated from only one culture flask.

4.2. N compounds and GS activity

4.2.1. Amino acids

The frozen samples were extracted with mortar and pestle with 10 mM ice-cold formic acid (Husted et al., 2000). Amino acids were separated with a HPLC system (Nova-Pak C_{18} bonded silica guard column and AccQ-Tag separation column, both from Waters), after derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl-carbamate (AQC) according to van Wandelen and Cohen (1997).

4.2.2. GS activity

The frozen samples were extracted with mortar and pestle on ice with 0.1 mM imidazole, pH 7.8, 0.5% (v/v) β-mercaptoethanol, 10% (w/v) glycerol, 0.33 M sucrose, 1.5% (w/v) polyvinyl polypyrrolidone and 1 mM phenylmethanesulfonyl fluoride. GS (EC. 6.3.1.2) activity was measured as synthetase reaction (O'Neil and Joy, 1975) by formation of γ-glutamylhydroxamate in 50 mM Tris–HCl, pH 7.5, containing 100 mM (*S*)-glutamate, 50 mM $MgSO_4$, 5 mM $NH_2OH \cdot HCl$ and 20 mM ATP. The reaction was terminated after 20 min at 37 °C with acidic $FeCl_3$ and quantified spectrophotometrically at 540 nm.

4.2.3. Total soluble protein

Total soluble protein in the GS extracts was estimated according to Bradford (1976) with bovine serum albumin as standard.

4.3. Analyses of betaxanthins

4.3.1. Extraction of betaxanthins

The freeze-dried sample was pulverized with mortar and pestle and 65 mg was extracted in darkness with 15 ml petrol for 30 min. The supernatant was discarded and the residue was extracted with aqueous MeOH (14 ml MeOH and 2 ml H_2O) in darkness for 30 min. After evaporation of the extract at 30 °C under vacuum the residue was dissolved in 200 μl solvent A (see below) and centrifuged. The supernatant was filtrated (Chromafil A-45, Macherey–Nagel, Düren, Germany; pore size 0.45 μm) prior to HPLC analysis.

4.3.2. Preparation of betaxanthin standards

Reference standards were prepared as described (Hempel and Böhm, 1997) by the release of betalamic acid from betaxanthins, excessive addition of the respective amino acid or dopamine and by chromatographic separation of the reaction product.

4.3.3. HPLC, characterization and quantification of betaxanthins

Forty or sixty microliters of betaxanthin extracts was injected onto a Li Chrospher 100 RP-18 endcapped column (250×4 mm, particle size 5 μm) protected by a 5×4 mm guard column with the same stationary phase and situated in a Jasco apparatus (Jasco Labor- und Datentechnik, Groß-Umstadt, Germany). It was equipped with an online-degasser (Gastorr GT-103), an autosampler (AS-950), a gradient pump (PU-980), a ternary gradient unit (LG-980-02), a column oven, a diode array detector (MD-910) and a system manager software. Separations were carried out at 20 °C and a flow rate of 1 ml min^{-1} with solvent A (2% MeOH in 0.1% aq. H_3PO_4) for 2 min, followed by a 0–20% gradient of solvent B (0.1% aq. H_3PO_4 in MeOH) within 30 min and further an isocratic treatment for 18 min which was finally followed by 10 min equilibration with solvent A. The column effluents were scanned between 200 and 600 nm. They were recorded at 280, 470 and 405 or 540 nm. Individual betaxanthins were characterized by comparison of their retention times and absorption maxima with those of the reference substances. The program Borwin integrated peak areas as absorption units min^{-1}. Peak areas of the same extract were practically identical in successive runs and differed by a maximum of 10% in runs at longer time intervals.

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