Plant Cell, Tissue and Organ Culture **38:** 167–169, 1994.

Regulatory mechanisms of biosynthesis of betacyanin and anthocyanin in relation to cell division activity in suspension cultures

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Key words: Anthocyanin, betacyanin, cell division, *Phytolacca*, suspension culture, *Vitis*

Abstract

Regulatory mechanisms of betacyanin biosynthesis in suspension cultures of *Phytolacca americana* and anthocyanin in *Vitis* sp. were investigated in relation to cell division activity.

Betacyanin biosynthesis in *Phytolacca* cells clearly shows a positive correlation with cell division, as the peak of betacyanin accumulation was observed at the log phase of batch cultures. Incorporation of radioactivity from labelled tyrosine into betacyanin also showed a peak at early log phase. Aphidicolin, an inhibitor of DNA synthesis, and propyzamide, an antimicrotubule drug, reduced betacyanin accumulation and inhibited the incorporation of radioactivity from labelled tyrosine into betacyanin at concentrations which were inhibitory to cell division. Both inhibitors reduced the incorporation of radioactivity from labelled tyrosine to 3,4-dihydroxyphenylalanine (DOPA), but the incorporation of labelled DOPA into betacyanin was not affected. These results suggest that the conversion of tyrosine to DOPA is coupled with cell division activity.

In contrast, the anthocyanin accumulation in *Vitis* cells showed a negative correlation with cell division. Accumulation occurred at the stationary phase in batch cultures when cell division ceased. Aphidicolin or reduced phosphate concentration induced a substantial increase in anthocyanin accumulation as well as the inhibition of cell division. Chalcone synthase (CHS) activity increased at the time of anthocyanin accumulation. Northern blotting analysis indicated that changes in CHS mRNA levels corresponded to similar changes in enzymatic activity. The pool size of endogenous phenylalanine was low during active cell division, but increased before anthocyanin began to accumulate and concomitantly with increasing levels of CHS mRNA. Exogenous supply of phenylalanine at the time of low endogenous levels induced the elevation of CHS mRNA and anthocyanin accumulation. These results indicate that the elevation of endogenous phenylalanine levels, when cell division ceases, may cause the increase in CHS mRNA levels, resulting in increased CHS activity and subsequently in anthocyanin accumulation in *Vitis* suspension cultures.

Abbreviations: CHS – chalcone synthase, CHFI – chalcone flavanone isomerase, DOPA – 3,4-dihydroxyphenylalanine, PAL – phenylalanine ammonia lyase

Introduction

Most secondary metabolites such as anthocyanins are accumulated during the stationary phase in batch suspension cultures. On the other hand, some secondary metabolites such as betacyanins are accumulated at the log phase (Sakuta et al. 1986). These facts suggest that biosynthesis of the former is correlated negatively with cell division, while that of the latter is correlated positively with cell division. In this paper, we investigated the regulatory mechanisms of biosynthesis of metabolites showing these two different accumulation

patterns in batch suspension cultures. Biosynthesis of anthocyanin in suspension cultures of *Vitis* sp. was investigated as an example of the former type of accumulation pattern and that of betacyanin in *Phytolacca americana* suspension cultures as an example of the latter one.

Results and discussion

Regulatory mechanisms of anthocyanin biosynthesis in Vitis sp. suspension cultures

Suspension cultures of *Vitis* used in these experiments were cultured at 27 °C in the dark. Cells reached the stationary phase 7 days after transfer and the accumulation of anthocyanins occurred 8 days after transfer, that is, after cessation of cell division. When cell division was inhibited by aphidicolin, an inhibitor of DNA synthesis, or by reduction of the phosphate concentration in media, a rapid accumulation of anthocyanins occurred coinciding with the cessation of cell division (Hirose et al. 1990). Changes in activities of three enzymes involved in the biosynthesis of anthocyanins were investigated. The activities of PAL and CHS increased to high levels immediately after transfer to fresh medium, but decreased thereafter and remained at very low levels during the logarithmic phase. When cell division ceased, activities of these two enzymes increased and high levels of activities remained during the accumulation of anthocyanins.

The activity of CHFI did not show the increase by the transfer effect and remained at a low level in the logarithmic phase, but increased when anthocyanins accumulated.

Changes in mRNAs of PAL and CHS were investigated by northern analysis. Two hybridization signals, (PAL1 and PAL2) were detected for cDNA of PAL. Both mRNAs encoding PAL were induced in the first day after transfer, but the amounts of them decreased immediately thereafter and remained at very low levels. When cell division ceased, the amounts of them increased again. The CHS mRNA showed a similar pattern of change in its amount as that of PAL. Since changes in amounts of PAL and CHS mRNAs were coincident with those of activities of PAL and CHS, it is suggested that the activities of these enzymes may be controlled at the transcriptional level.

The size of the endogenous pool of phenylalanine was small when active cell division occurred, but it increased rapidly just before increased accumulation of anthocyanin (8 days after transfer). Noé et al. (1980) demonstrated that the pool of endogenous phenylalanine, which was enlarged as a result of treatment with L-AOPP, an inhibitor of PAL, induced PAL activity. It is possible, therefore, that in *Vitis* cultures an elevated level of endogenous phenylalanine may induce PAL and CHS activities. In order to examine this possibility, phenylalanine was added exogenously to the media 4 days after transfer when cell division was occurring actively and anthocyanins were not accumulated. Phenylalanine added at 1 and 5 mM induced more rapid increases of accumulation of anthocyanin than controls in which diluted water was added, but the effect of 5 mM phenylalanine was more pronounced. Exogenous phenylalanine added 4 days after transfer also induced increases in the activities of PAL, CHS and CHFI. Northern analysis revealed that when phenylalanine (5 mM) was added 4 days after transfer, mRNAs of PAL1 and 2 and CHS could be detected after 5 days (1 day after the addition) and the amounts of the mRNAs reached their maxima after 6 days, whereas in the control culture mRNAs of PAL and CHS were detected only after 8 days and reached their maxima after 10 days of culture, though a transient expression of the mRNAs was observed just after transfer. We investigated the induction of expression of CHS mRNA by phenylalanine in more detail by northern analysis and found that CHS mRNA could be detected shortly after addition of phenylalanine, even after 1 hr. The amino acids leucine and tyrosine had no effect on the induction of accumulation of anthocyanins.

From the results obtained, a possible mechanism for the regulation of anthocyanin biosynthesis during batch suspension cultures of *Vitis* cells is proposed. When cells divide actively the endogenous level of phenylalanine is low, because phenylalanine is utilized for biosynthesis of protein. When the cell division ceases, the level of endogenous phenylalanine increases and acts as a trigger for the initiation of transcription of PAL and CHS mRNA, resulting in de novo synthesis of PAL and CHS proteins and subsequently in the increase of the activities of these enzymes, which leads to the accumulation of anthocyanins. The increased level of endogenous phenylalanine also may supply more precursors for the biosynthesis of anthocyanins. Thus the accumulation of anthocyanins shows a negative correlation with cell division in batch suspension cultures of *Vitis*.

Regulatory mechanisms of betacyanin biosynthesis in Phytolacca americana suspension cultures

As we reported previously (Sakuta et al. 1986), betacyanins accumulate in the logarithmic phase. In contrast to most other secondary metabolites, such as anthocyanins, the maximum betacyanin content per cell was observed 5 days after transfer of *Phytolacca americana* suspension cultures. We investigated whether not only accumulation but also biosynthesis of betacyanins occurred during active cell division using labelled tyrosine. Tracer experiments revealed clearly that the peak of incorporation of radioactivity from tyrosine to betacyanin was observed 4 days after transfer, at the early log phase. When aphidicolin, an inhibitor of DNA synthesis, was added to suppress the cell division, the accumulation of betacyanin as well as its biosynthetic activity were completely suppressed. When the cell division was inhibited by propyzamide, an antimicrotubule drug, the biosynthesis of betacyanins from tyrosine was also inhibited. These results indicate that the biosynthesis of betacyanins is associated not only with DNA synthesis, but also with mitosis.

Betacyanin is believed to be synthesized from tyrosine via DOPA. We examined the incorporation of radioactivity from labelled tyrosine or DOPA to betacyanin in the presence or absence of aphidicolin or propyzamide in order to elucidate which step of biosynthesis of betacyanin is coupled with cell division. The results obtained revealed that the incorporation of radioactivity from tyrosine to betacyanins was inhibited remarkably by both inhibitors, while that from DOPA was not. It is, therefore, concluded that the step from DOPA to betacyanins is not coupled with cell division, but that from tyrosine to DOPA is coupled.

Conclusions

The regulatory mechanisms of biosynthesis of anthocyanins, which shows a negative correlation with cell division, and of betacyanins, which shows a positive correlation, were investigated in relation to cell division using *Vitis* sp. and *Phytolacca americana* suspension cultures, respectively. Concerning the regulatory mechanisms of anthocyanin biosynthesis, it is concluded that cessation of cell division causes the increase of pool size of endogenous phenylalanine which may trigger the transcription of mRNAs of enzymes involved in anthocyanin biosynthesis such as PAL and CHS, resulting in increased activities of those enzymes and in increased accumulation of anthocyanins. Thus, the accumulation of anthocyanin shows a negative correlation with cell division.

Concerning the regulatory mechanisms of biosynthesis of betacyanins, it was believed that the biosynthesis of betacyanins is not only associated with DNA synthesis but also with mitosis. It was concluded that the step of hydroxylation of tyrosine to DOPA is coupled with cell division, but the steps from DOPA to betacyanins are not. However, it still remains to be solved what event(s) of cell division is coupled or associated with hydroxylation of tyrosine to DOPA.

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