Studies on alternative splicing in higher plants

Shoji Mano

DOCTOR OF SCIENCE

Department of Molecular Biomechanics
School of Life Science
The Graduate University for Advanced Studies

1998

博士論文の要旨

平成10年1月9日

出願者
生命科学研究科　分子生物機構論専攻
ふりがな　ま　の　しょうじ
氏名　真野　昌二
(学籍番号)　953204

学位申請論文

題目　Studies on alternative splicing in higher plants

Alternative splicing has emerged in recent years as a widespread mechanism for regulating gene expression and generating isoform diversity. It has been a subject of major interest, both in its own right as an important biological regulatory mechanism, and also because it has provided insights into some fundamental aspects of splicing. The analysis of alternative splicing has been mainly performed in mammals and some important information about the mechanism such as the identifications of *trans* factors and *cis* regulatory elements were obtained. However, recent studies demonstrated the involvement of alternative splicing in the synthesis of some enzymes in plants. I analyzed about alternative splicing of two enzymes in pumpkin. One is hydroxypyruvate reductase (HPR) that is known for a leaf peroxisomal enzyme and the other is chloroplastic ascorbate peroxidase (APX).

From the two kinds of cDNA cloning for HPR and the determination of their nucleotide and genomic sequences, it has been proposed that alternative splicing could function in the synthesis of their mRNAs [1]. Therefore, I tried to detect the presence of two HPR proteins in pumpkin cells. The immunofluorescent microscopy using an antibody against HPR showed that HPR1 and HPR2 proteins are localized in leaf peroxisomes and the cytosol, respectively. These localizations were confirmed by the analysis using transgenic plants that expressed fusion proteins with green fluorescent protein. Based on these results and the nucleotide and deduced amino acid sequences, it was concluded that alternative splicing controlled the production of the targeting signal to peroxisomes, resulting in the determination of two HPR proteins. That is, a single HPR gene has two

pairs of the GT-AG doublets in the region encoding the carboxy terminus, and the way to splice affects the presence of the targeting signal. The immunoblot analysis revealed that the accumulations of two electrophoretically similar polypeptides corresponding to HPR1 and HPR2 proteins were increased during germination of pumpkin cotyledons and enhanced by light. Moreover, the RT-PCR analysis showed that this light induction shifted the splicing pattern from the production of almost equal amounts of HPR1 and HPR2 mRNAs to mainly production of HPR2 mRNA. These results suggest that alternative splicing of HPR pre-mRNA is regulated developmentally and by light [2]. The existence of only HPR2 in stamen tissues in addition to the fact of the much induction of HPR2 protein compared to HPR1 provides the interest of the physiological role of HPR2, but the function of HPR2 protein in the cytosol remains unclear.

To date, cDNAs for HPR in higher plants have been cloned from pumpkin [1] and cucumber [3]. HPR cDNA in cucumber encodes the protein without the targeting signal, namely, the HPR2-type protein. However, it was indicated that another HPR protein with the targeting signal to microbodies existed in cucumber, because the cucumber HPR gene also has two pairs of the GT-AG doublets at the same position of pumpkin HPR gene. It is therefore important to examine whether another plants have the HPR proteins. Five *Arabidopsis* EST clones homologous to pumpkin HPR have been registered at the Arabidopsis Biological Resource Center (ABRC) at Ohio State University. The determination of their nucleotide sequences encoding the carboxy termini revealed that all EST clones encode the HPR1-type protein. It was confirmed that only one polypeptide was recognized by antibodies against HPR. At the result of the genomic sequence, *Arabidopsis* HPR gene has one kind of the GT-AG doublet, although the HPR gene exists as a single copy like does pumpkin HPR gene. These results show that alternative splicing does not undergo in *Arabidopsis* [4]. The immunoblot analysis demonstrated that pumpkin and cucumber, of tested plants, seem to have two kinds of HPR proteins, whereas other plants seem to have one HPR protein, indicating that all plants do not necessarily need the cytosolic HPR.

Plant cells have four kinds of APX. Of these, two APXs are localized in the stroma (sAPX) and on the thylakoid membrane (tAPX) in chloroplasts, and they are considered as the scavengers of hydrogen peroxide. From the partial sequence at the amino termini and the biochemical properties, it

was speculated in our laboratory that sAPX and tAPX might be produced by alternative splicing. Therefore, I tried to isolate sAPX cDNA clone in pumpkin and determine its nucleotide sequence. As compared with the sequence of pumpkin tAPX cDNA [5], sAPX cDNA showed the complete identity except the region encoding the carboxy domain that contained the thylakoid membrane spanning region. The analysis of genomic structure clarified the presence of one donor site and two acceptor sites, indicating that the way to use each acceptor site determined the inclusion of the thylakoid membrane spanning region. The immunoblot analysis revealed that this alternative splicing are also regulated developmentally and by light. Moreover, the regulation of tissue-specific manner is present [6].

At the results of these two examples, it was demonstrated that alternative splicing which produces variants whose subcellular or suborganellar localizations are different exists in higher plants. Interestingly, this alternative splicing showed the light-dependency and the tissue-specific manner. Tissue specificity is the phenomena as same as seen in alternative splicing in mammals. However, the light-dependency is specific to plants, indicating the presence of the novel mechanism of the signal transduction. Also, these data suggest that the conversions of microbodies (from glyoxysomes to leaf peroxisomes) and chloroplasts (from etioplasts to chloroplasts) are closely related to the regulation of HPR and chloroplastic APXs by alternative splicing, respectively.

[1] Hayashi M., Tsugeki R., Kondo M., Mori H. and Nishiura M. (1996) *Plant Mol. Biol.*, 30, 183-189.

[2] Mano S., Hayashi M. and Nishimura M. (1998) *Mol. Cell Biol.*, (submitted)

[3] Greenler J. M., Sloan J. S., Schwartz B. W. and Becker W. M. (1989) *Plant Mol. Biol.*, 13, 139-150.

[4] Mano S., Hayashi M., Kondo M. and Nishimura M. (1997) *Plant Cell Physiol.* 38, 449-455.

[5] Yamaguchi K., Hayashi M. and Nishimura M. (1996) *Plant Cell Physiol.* 37, 405-409.

[6] Mano S., Yamaguchi., Hayashi M. and Nishimura M. (1997) *FEBS Lett.* 413, 21-26.

Contents

Abbreviations 1

Abstract 2

Chapter 1 General introduction 5

1.1. RNA processing 6

1.1.1. Gene expression regulated by alternative splicing 7

1.1.2. Protein localization controlled by alternative splicing 9

1.1.3. Mechanism of alternative splicing 10

1.1.4. Alternative splicing in higher plants 12

1.2. Protein transport into organelle 13

1.2.1. Microbody-transition controlled by light 13

1.2.2. Targeting signals into microbodies 16

1.3. Hydroxypyruvate reductase 17

1.4. Ascorbate peroxidase 20

1.5. Aim of this study 22

Chapter 2 Alternative forms of pumpkin hydroxypyruvate reductase: identification and characterization 24

2.1. Summary 25

2.2. Introduction 25

2.3. Materials and methods 26

2.4. Results 31

2.5. Discussion 46

Chapter 3 Molecular cloning and characterization of hydroxypyruvate reductase in *Arabidopsis thaliana* 49

3.1. Summary 50

3.2. Introduction 50

3.3. Materials and methods 51

3.4. Results 53

3.5. Discussion 64

Chapter 4 Molecular cloning and characterization of an alternatively spliced chloroplastic ascorbate peroxidase in pumpkin, stromal ascorbate peroxidase 66

4.1. Summary 67

4.2. Introduction 67

4.3. Materials and methods 68

4.4. Results 70

4.5. Discussion 81

Chapter 5 General discussion 84

Summary 85

General discussion 88

References 92

Acknowledgments 100

Abbreviations

AGPase	ADP-glucose pyrophosphorylase
APX	ascorbate peroxidase
BSA	bovine serum albumin
BY-2	Tobacco Bright Yellow No. 2 suspension cultured cells
APX	cytosolic APX
EDTA	ethylenediaminetetraacetic acid
ESE	exonic splicing enhancer
EST	expressed sequence tag
FITC	fluorescein isothiocyanate
GFP	green fluorescent protein
GUS	β-glucuronidase
HPR	hydroxypyruvate reductase
kb	kilo base
kD	kilo dalton
mbAPX	microbody membrane-bound APX
PBS	phosphate buffered saline
PMSF	phenylmethanesulfonyl fluoride
pre-mRNA	precursor of mRNA
PTS	peroxisomal targeting signal
RT-PCR	reverse transcription of RNA followed by PCR
sAPX	stromal APX
SDS	sodium dodecylsulfate
sGFP	synthetic GFP
snRNA	small nuclear RNA
snRNP	small nuclear ribonucleoprotein
SSC	standard saline citrate
tAPX	thylakoid membrane-bound APX
Tris	2-amino-2-hydroxymethyl-1,3-propanediol

Abstract

Alternative splicing has emerged in recent years as a widespread mechanism for regulating gene expression and generating isoform diversity. It has been a subject of major interest, both in its own right as an important biological regulatory mechanism, and also because it has provided insights into some fundamental aspects of splicing. The analysis of alternative splicing has been mainly performed in mammals and some important information about the mechanism such as the identifications of *trans* factors and *cis* regulatory elements were obtained. However, recent studies demonstrated the involvement of alternative splicing in the synthesis of some enzymes in plants. I analyzed about alternative splicing of two enzymes in pumpkin. One is hydroxypyruvate reductase (HPR) that is known for a leaf peroxisomal enzyme and the other is chloroplastic ascorbate peroxidase (APX).

From the two kinds of cDNA cloning for HPR and the determination of their nucleotide and genomic sequences, it has been proposed that alternative splicing could function in the synthesis of their mRNAs (Hayashi et al., 1996). Therefore, I tried to detect the presence of two HPR proteins in pumpkin cells. The immunofluorescent microscopy using an antibody against HPR showed that HPR1 and HPR2 proteins are localized in leaf peroxisomes and the cytosol, respectively. These localizations were confirmed by the analysis using transgenic plants that expressed fusion proteins with green fluorescent protein. Based on these results and the nucleotide and deduced amino acid sequences, it was concluded that alternative splicing controlled the production of the targeting signal to peroxisomes, resulting in the determination of two HPR proteins. That is, a single HPR gene has two pairs of the GT-AG doublets in the region encoding the carboxy terminus, and the way to splice affects the presence of the targeting signal. The immunoblot analysis revealed that the accumulations of two electrophoretically similar polypeptides corresponding to HPR1 and HPR2 proteins were increased during germination of pumpkin cotyledons and enhanced by light. Moreover, the RT-PCR analysis showed that this light induction shifted the splicing pattern from the production of almost equal amounts of HPR1 and HPR2 mRNAs to mainly production of HPR2

mRNA. These results suggest that alternative splicing of HPR pre-mRNA is regulated developmentally and by light. The existence of only HPR2 protein in stamen tissues in addition to the fact of the much induction of HPR2 protein compared to HPR1 protein provides the interest of the physiological role of HPR2 protein, but the function of HPR2 protein in the cytosol remains unclear.

To date, cDNAs for HPR in higher plants have been cloned from pumpkin (Hayashi et al., 1996) and cucumber (Greenler et al., 1989). HPR cDNA in cucumber encodes the protein without the targeting signal, namely, the HPR2-type protein. However, Hayashi et al. (1996) indicated the presence of another HPR protein with the targeting signal in cucumber, because the cucumber HPR gene also has two pairs of the GT-AG doublets at the same position of pumpkin HPR gene. It is therefore important to examine whether another plants have the HPR proteins. Five *Arabidopsis* EST clones homologous to pumpkin HPR have been registered at the Arabidopsis Biological Resource Center (ABRC) at Ohio State University. The determination of their nucleotide sequences encoding the carboxy termini revealed that all EST clones encode the HPR1-type protein. It was confirmed that only one polypeptide was recognized by antibodies against HPR. At the result of the genomic sequence, *Arabidopsis* HPR gene has one kind of the GT-AG doublet, although the HPR gene exists as a single copy like does pumpkin HPR gene. These results show that alternative splicing does not undergo in *Arabidopsis*. The immunoblot analysis demonstrated that pumpkin and cucumber, of tested plants, seem to have two kinds of HPR proteins, whereas another plants seem to have one HPR protein, indicating that all plants do not necessarily need the cytosolic HPR.

Plant cells have four kinds of APX. Of these, two APXs are localized in the stroma (sAPX) and on the thylakoid membrane (tAPX) in chloroplasts, and they are considered as the scavengers of hydrogen peroxide. From the partial sequence at the amino termini and the biochemical properties, it was speculated in our laboratory that sAPX and tAPX might be produced by alternative splicing. Therefore, I tried to isolate sAPX cDNA clone in pumpkin and determine its nucleotide sequence. As compared with the sequence of pumpkin tAPX cDNA (Yamaguchi et al., 1996), sAPX cDNA showed

the complete identity except the region encoding the carboxy domain that contained the thylakoid membrane spanning region. The analysis of genomic structure clarified the presence of one donor site and two acceptor sites, indicating that the way to use each acceptor site determined the inclusion of the thylakoid membrane spanning region. The immunoblot analysis revealed that this alternative splicing are also regulated developmentally and by light. Moreover, the regulation of tissue-specific manner is present.

At the results of these two examples, it was demonstrated that alternative splicing which produces variants whose subcellular or suborganellar localizations are different exists in higher plants. Interestingly, this alternative splicing showed the light-dependency and the tissue-specific manner. Tissue specificity is the phenomena as same as seen in alternative splicing in mammals. However, the light-dependency is specific to plants, indicating the presence of the novel regulation of alternative splicing. Also, these data suggest that the conversions of microbodies (from glyoxysomes to leaf peroxisomes) and chloroplasts (from etioplasts to chloroplasts) are closely related to the regulation of HPR and chloroplastic APXs by alternative splicing, respectively.

Chapter 1
General introduction

1.1. RNA processing

Genes in eukaryotes are often interrupted by intervening sequences (introns) that must be removed during gene expression. Transcribed pre-mRNAs undergo a series of modifications in the nucleus in order to function as mature RNAs, namely, the addition to 5' capping with 7-methyl guanosine, the addition of poly(A) tail to the 3' end of nascent transcripts and the removal of introns (RNA splicing). RNA splicing is the mechanism by which introns are precisely removed and the flanking functional sequences (exons) are ligated. In order to avoid disrupting the open reading frame, precise removal of introns is required, and the analysis about the mechanism, such as *trans* factors and *cis*-splicing elements, have been carried out (Padgett et al., 1986). With respect to *cis*-splicing elements, it was recognized that sequences at the boundaries between introns and exons are not random, namely, introns begin with GT and end with AG (the GT-AG rule). A number of available splice junction sequences revealed that this GT-AG rule was obeyed and that a somewhat longer consensus sequence could be found (Mount, 1982). /GUAUGU (the vertical line represents the exon-intron border) is identified as the 5' splice site (the donor site) (Parker and Guthrie, 1985; Siliciano and Guthrie, 1988), whereas YnNCAG/G (the vertical line represents the intron-exon border) is the consensus sequence as the 3' splice site (the acceptor site) (Ohsima and Gotoh, 1987). In addition, the polypyrimidine tract, which plays a role of 5' cleavage reaction, and the branch point sequence are determined as the important sequences for RNA splicing. The branch point sequence, UACUAAC, is highly conserved and the final A in the sequence is the site of branch formation (Padgett et al., 1986). However, recent study revealed the presence of another boundaries, whose introns begin with AT and end with AG (the AT-AG rule), and the identification of some components which bind to such splice sites (Tarn and Steitz, 1997). Splicing occurs in a large ribonucleoprotein complex, spliceosome, which consists of five major small nuclear RNAs (U1, U2, U4, U5 and U6 snRNAs) and ribonucleoproteins (snRNPs) that contain one or a few snRNRs, and unidentified proteins. In the case of splicing, a two-step reaction involving successive transesterification reactions is occurred as the followings. Initially, U1 snRNP and U2

snRNP bind to the 5' splice donor site and the branch point region in pre-mRNA, respectively, and then the cleavage occurs at the 5' donor splice site. The 5' end of the intron is joined to an adenosine residue located within the intron, giving rise to a lariat form of the intron. Then the binding of the U4-U5-U6 snRNP complex happens, resulting in ligation of the exons and release of the lariat intron (Smith et al., 1989; Madhani and Guthrie, 1994). All components and *cis*-elements involving in RNA splicing have not been completely identified and some studies about the elucidation of RNA splicing are in progress. This splicing is called general (or constitutive) splicing and is distinguished from alternative splicing. The modifications including RNA splicing occur in the nucleus and processed RNAs get out from the nucleus to the cytosol.

1.1.1. Gene expression regulated by alternative splicing

Alternative splicing is a well-known post-transcriptional device for regulating gene expression and generating isoform diversity in eukaryotic organisms. This device gives rise to protein isoforms sharing extensive regions of identity and varying only in specific domains without permanent gene rearrangement or changes in transcriptional activity, resulting in the advantages as a level of qualitative and quantitative regulation of gene expression, highlighting the importance of alternative splicing as a post-transcriptional level of gene regulation as well as the transcriptional level. Figure 1-1 summarizes the classification for alternative splicing based on the various combinations of different promoters, different splice sites and different poly(A) sites. They are various patterns that are from the simplest form of splice/don't splice to the complicate form of mutually exclusive exons (McKeown, 1992). This mechanism can involve on/off regulation of the products for particular genes or it can produce alternative products which allow for protein function, ranging from the determination of cellular and subcellular localization to the modulation of enzyme activity. Alternative splicing is also used to quantitatively regulate gene expression by producing prematurely truncated open reading frames or by regulating mRNA stability or translational efficiency via variability in the untranslated

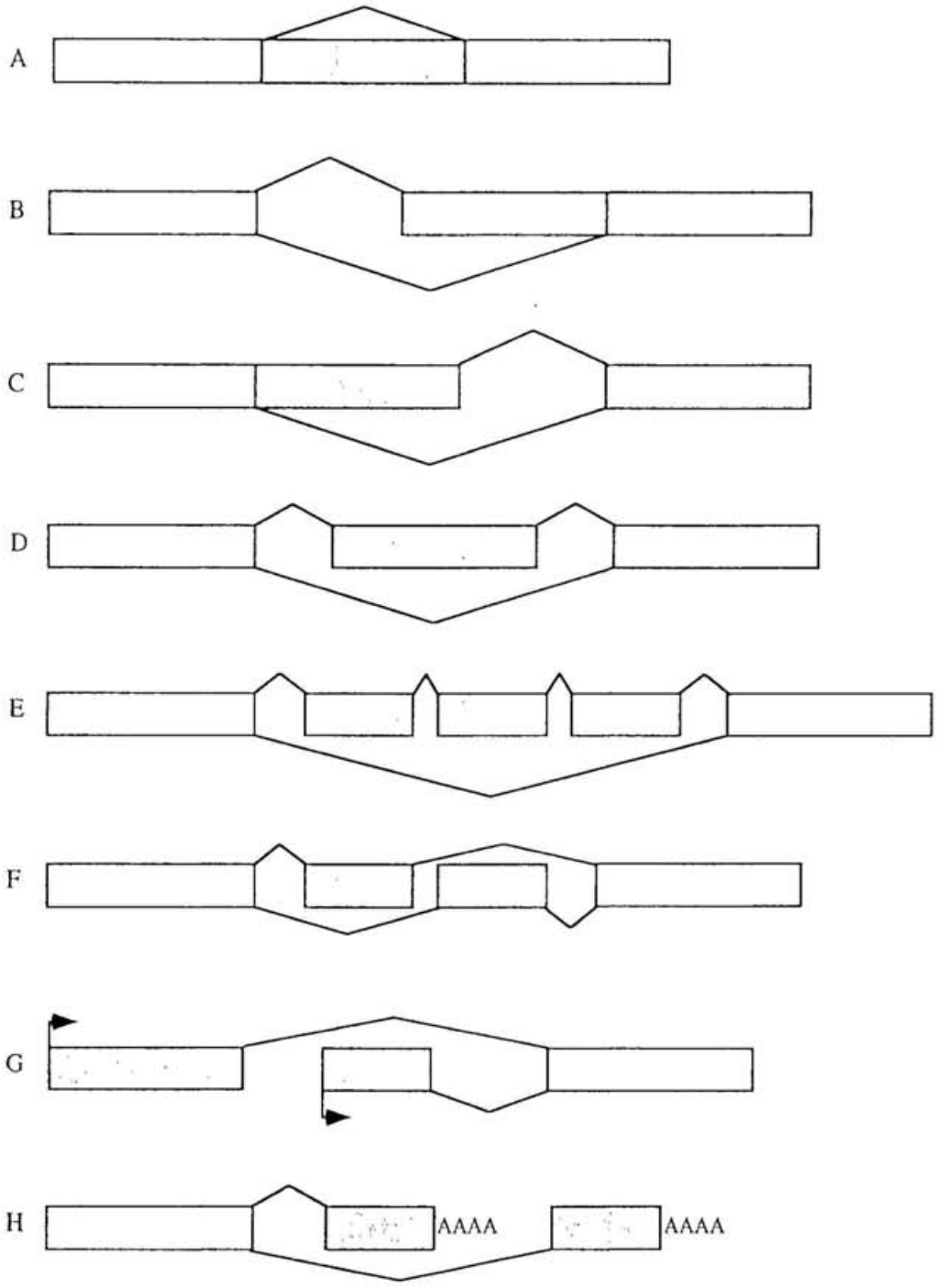


Figure 1-1. Various forms of alternative splicing for pre-mRNA. (A) Splice / don't splice, (B) Alternative 3' splice sites, (C) Alternative 5' splice sites, (D) Exon skipping, (E) Multiple exon skipping, (F) Mutually exclusive exons, (G) Alternative promoters / alternative 5' splice sites, (H) Alternative poly A sites / alternative 3' splice sites (MacKeown, 1992).

regions. It has been mainly reported in mammals that the number of alternatively spliced genes has expanded enormously (Inoue et al., 1990; 1992; Corti et al., 1994; Arsura et al., 1995; Duncan et al., 1995; Lui et al., 1996; Miyatake et al., 1996; Sajjadi et al., 1996; Si et al., 1996; Sugiyama et al., 1996; Walker et al., 1996; Wetering et al., 1996; Zhao and Manley, 1996; Yang et al., 1996; Yu and Toole, 1996; Diamant et al., 1997; Lievens et al., 1997; Schischmanoff et al., 1997; Han et al., 1997). In all cases, alternative splicing produces some variants from single genes, resulting in the diversity of the function and subcellular localization. In higher plants, recent studies demonstrated the involvement of alternative splicing in the synthesis of some proteins (Chapter 1.1.4).

1.1.2. Protein localization controlled by alternative splicing

As stated above, alternative splicing gives various functional effects to different products. In the case that spliced regions are involved in the determination of the cellular or subcellular localization, such as the targeting sequences, alternatively spliced products reveal different localizations. For example, carnitine acetyltransferases, which catalyzes the reversible transfers short-chain acyl groups from acyl-coenzyme As to carnitine, are localized in mitochondria and peroxisomes (Corti et al., 1994). In the case of the deletion of the leader sequence to mitochondria at the amino terminal region, carnitine acetyltransferase is transported to peroxisomes due to the peroxisomal targeting signal at the carboxy terminus, whereas alternatively spliced carnitine acetyltransferase, which has the leader sequence at the amino terminal region, is localized in mitochondria in spite of the existence of the peroxisomal targeting signal, because the leader sequence to mitochondria is more prevailing than the peroxisomal targeting signal. Murine CD44 also utilizes alternative splicing to generate numerous isoforms (Yu and Toole, 1996). This protein is a multifunctional cell-surface glycoprotein that is expressed as multiple isoforms derived from a single gene. Some of them are truncated prior to the transmembrane domain, resulting in the secretion as soluble proteins. The membrane-bound glycoprotein gp130, which acts as an affinity converting and signal transducing

receptor for interleukin-6 and several other cytokines, undergoes alternative splicing, leading to a frame-shift which produces a stop codon before the transmembrane domain. At a result, truncated gp130 is produced as a soluble form and secreted (Diamant et al., 1997).

1.1.3. Mechanism of alternative splicing

How does alternative splicing occur in the nucleus? In mammals, alternative splicing often shows the tissue-specific (Miyatake et al., 1996; Sajjadi et al., 1996; Si et al., 1996; Sugiyama et al., 1996; Yang et al., 1996; Yu and Toole, 1996; Zhao and Manley, 1996; Han et al., 1997; Lievens et al., 1997) or the developmental stage-specific manners (Lui et al., 1996; Schischmanoff et al., 1997; Walker et al., 1996), although the mechanism is still obscure. From the analysis of the somatic sex determination in *Drosophila melanogaster*, which is the best characterized example, a lot of important information about the mechanism of alternative splicing were obtained (Bell et al., 1988; Baker, 1989; Inoue et al., 1990; 1992; Hoshijima et al., 1991,; Sakamoto et al., 1992). In *Drosophila,* the somatic sex is determined by the ratio of X chromosomes to autosomes, namely, one X chromosomes and two sets of autosomes are male, whereas individuals with equal numbers of X chromosomes and sets of autosomes are female. Many components, including sex-lethal (*sxl*), transformer (*tra*), transformer-2 (*tra-2*) and double-sex (*dsx*) products, consist of a hierarchy of regulatory pathway for the sex determination regulated by alternative splicing. In the case of individuals with equal numbers of X chromosomes and sets of autosomes (female form), exon 3 skipping is caused by alternative splicing during the maturation of *sxl* pre-mRNA, leading to the production of active Sxl protein. Sxl protein controls alternative splicing of *tra* pre-mRNA by the binding the upstream region of exon 2, resulting in exon 2 skipping. At a result, active Tra protein is produced. Tra protein along with another factor, Tra-2 protein, regulates alternative splicing of *dsx* pre-mRNA by the binding in the exon 4, causing the inclusion of exon 4. This series of events ends up to the determination of female. In the

case of male, however, active Sxl protein is not produced because of inclusion of exon 3 that contains a stop codon, giving rise to truncated Tra protein. Truncated Tra protein can not bind the exon 4 of *dsx* pre-mRNA so that exon 4 skipping happens, leading to the determination of male. The binding sites of Sxl, Tra and Tra-2 proteins have been determined in each pre-mRNA (Inoue et al., 1990; 1992; Hoshijima et al., 1991; Sakamoto et al., 1992). It has been reported that alternative splicing appears to be achieved by subtle variations of the general splicing mechanism described previously (Smith et al., 1989). Moreover, recent analyses revealed the presence of additional intronic and exonic *cis*-elements for alternative splicing. Huang and Spector (1996) demonstrated intron-dependent splicing of pre-mRNA. The exonic splicing enhancer(s) (ESE), which consists of contiguous repeats of the motif of GARGAR (R indicates purine), are considered as the *cis*-element(s) for binding of *trans* factors such as SR proteins (Gontarek and Derse, 1996; Yeakley et al., 1996; Gallego et al., 1997; Tacke et al., 1997). SR proteins, which have one or two RNA recognition motif(s) at the amino terminal region and the Arg and Ser residue-rich domain at the carboxy terminal region, are considered as candidates for *trans* regulating factors. It has been demonstrated that SR proteins can promote differential regulation and use of different splice sites by binding to the ESEs and interacting with another splicing factors or spliceosomal proteins (Zahler et al., 1992; Screaton et al., 1995; Chandler et al., 1996; Jumaa and Nielsen, 1997). Various SR proteins are expressed according to tissues and developmental stages whose molecular masses vary from 20 kDa to 75 kDa, and some of them appear to function as components of general splicing complexes (Valcárcel and Green, 1996). From the analyses of mammals, the ratio of SR proteins influences the alternative splicing that is related to the tissue specificity (Chandler et al., 1996; Gontarek and Derse, 1996; Screaton et al., 1995; Zahler et al., 1992). Tra and Tra-2 proteins belong to the SR protein family. In higher plants, Lazar et al. (1995) and Lopato et al. (1996) showed the existences of polypeptides that crossreact with antibodies against human SR protein in the extracts of *Arabidopsis*, tobacco cultured cells and carrot. Several homologues have been cloned only in *Arabidopsis*, and it has been proved that they could restore splicing in

HeLa S100 splicing-deficient extracts, indicating that they play a crucial role in alternative splicing (Lopato et al., 1996), suggesting the presence of the mechanism similar to mammals.

1.1.4. Alternative splicing in higher plants

Recent papers report on the occurrence of alternative splicing in higher plants as well as in mammals but only very few alternative spliced genes have been documented. In this section, I would like to introduce recent reports related to alternative splicing in higher plants. Three kinds of chorismate synthase, which catalyze the last step in the biosynthesis of the aromatic amino acids such as phenylalanine, tyrosine and tryptophan, exist. Of these, two proteins are synthesized by alternative splicing and the ratio of these two mRNAs differs from different organs in tomato (Görlach et al., 1995). The synthesis of 3-hydroxy-3-methylglutaryl coenzyme A reductases, which has a role of the synthesis of mevalonic acid, is regulated by the use of alternative promoters in *Arabidopsis* (Lumbreras et al., 1995). In the case of rice homeobox mRNA, alternative splicing, which shows the tissue-specific fashion, occurs at three different acceptor sites, producing three mRNAs from a single pre-mRNA (Tamaoki et al., 1995). H-protein is one of four components of a mitochondrial multi-enzyme complex of the glycine cleavage system and alternative splicing shows the regulation dependent on the species-specific manner, namely, alternative splicing of H-protein occurs in the C4 species and does not in any C3 and C3-C4 intermediate species (Kopriva et al., 1996). Moreover, the production of FCA, a protein controlling flowering time, and the homologue of *Brassica S*-receptor kinase is controlled by alternative splicing in *Arabidopsis* (Macknight et al., 1997; Tobias and Nasrallah, 1996). In both cases, ones are located in the cell surface because of the presence of the membrane-spanning domain, and the others are secreted on account of the deletion of this domain by alternative splicing. Gene of ADP-glucose pyrophosphorylase (AGPase) in barley has two exon 1 that are called exon 1a and exon 1b (Thorbjørnsen et al., 1996). Of these, exon 1b contains the transit peptide to amyloplasts. Alternative

promoter controls the selection of exon 1a or exon 1b. When the distal promoter, which is located at the upstream of exon 1a, is used, exon 1a is included, resulting in the production of cytosolic AGPase. When another promoter, which is present between exon 1a and exon 1b, is used, the inclusion of exon 1b leads to the production of the amyloplastic AGPase. Denyer et al. (1996) and Thorbjørnsen et al. (1996) estimated enzymatic activities of both AGPases in maize and barley endosperm, respectively, concluding that both AGPases function in the biosynthesis of ADP-glucose. In other cases described above, the physiological roles or functions of alternatively spliced variants remain unclear.

1.2. Protein transport into organelle

It is necessary for proteins to be transported correctly to a given place in order to function as active forms. The functioning of organelles is therefore strongly dependent on the import of many nuclear-encoded proteins. The targeting of proteins to an organelle in a eukaryotic cells is brought about by signals. On behalf of such signals, presequences for translocation to mitochondria (Roise and Schatz, 1988), transit peptides for translocation to chloroplasts (Karlin-Neumann and Tobin, 1986), retention signals in the endoplasmic reticulum (Pelham, 1989), targeting signals to vacuole (Nakamura and Matsuoka, 1993; Paris and Rogers, 1996) and the peroxisomal targeting signals (PTSs) have been identified. In the case of transport to microbodies (glyoxysomes and peroxisomes), two kinds of PTS have been identified. One is located at the carboxy terminus (PTS1) and the other is in the amino terminal presequence of the precursor protein (PTS2) (With respect to transport into microbodies, I describe in Chapter 1.2.2).

1.2.1 Microbody-transition controlled by light

Microbodies are ubiquitous organelles bound by a single membrane that exist in mammals, plants, fungi, yeast and invertebrate animals (Beevers, 1979). These

organelles contain hydrogen peroxide-producing enzyme and catalase that catalyzes the reduction of hydrogen peroxide to water. In higher plants, microbodies are subcategorized as glyoxysomes, leaf peroxisomes and unspecialized microbodies. Glyoxysomes, which are found in oil-rich tissues of etiolated cotyledons, contain some enzymes required for the β-oxidation of fatty acids and the glyoxylate cycle. Glyoxysomes play a major role in the mobilization of lipids that is necessary for the gluconeogenesis. Leaf peroxisomes, which are present in green leaves and cotyledons, contain enzymes of the glycolate cycle and participate in the photorespiration process (Figure 1-2). Unspecialized microbodies with undefined physiological function are present in other organs such as roots and stems. Some microbody enzymes have been cloned and characterized (Mori and Nishimura, 1989; Tsugeki et al., 1993; Kato et al., 1995; 1996; 1998; Mano et al., 1996). Activities and amounts of mRNAs for glyoxysomal enzymes, such as malate synthase, citrate synthase and isocitrate lyase, are induced in darkness during germination of oil-seed cotyledons like pumpkin, cucumber and watermelon. However, when seedlings grown in darkness are transferred to continuos light, activities of glyoxysomal enzymes are dramatically decreased, whereas activities of leaf peroxisomal enzymes, such as glycolate oxidase and hydroxypyruvate reductase, are increased, showing the functional transformation of microbodies. Nishimura et al. (1993) and Titus and Becker (1985) demonstrated that glyoxysomes are transformed directly to leaf peroxisomes and that this microbody transition is controlled by light. Moreover, Schantz et al. (1995) reported the implication of the signal transduction regulated by phytochrome. Degradation of glyoxysomal enzymes, prevention of transport and gene expression of glyoxysome-specific enzymes in addition to activation of syntheses of leaf peroxisomal enzymes are involved in the mechanism of this microbody-transition from glyoxysomes to leaf peroxisomes. On the contrary, the conversion from leaf peroxisomes to glyoxysomes occurs in senescing and detached leaves (Nishimura et al., 1993). In this case, leaf peroxisomal enzymes disappear and activities of glyoxysomal enzymes are induced again. At present, the mechanism of the reverse microbody-transition remains to be determined.

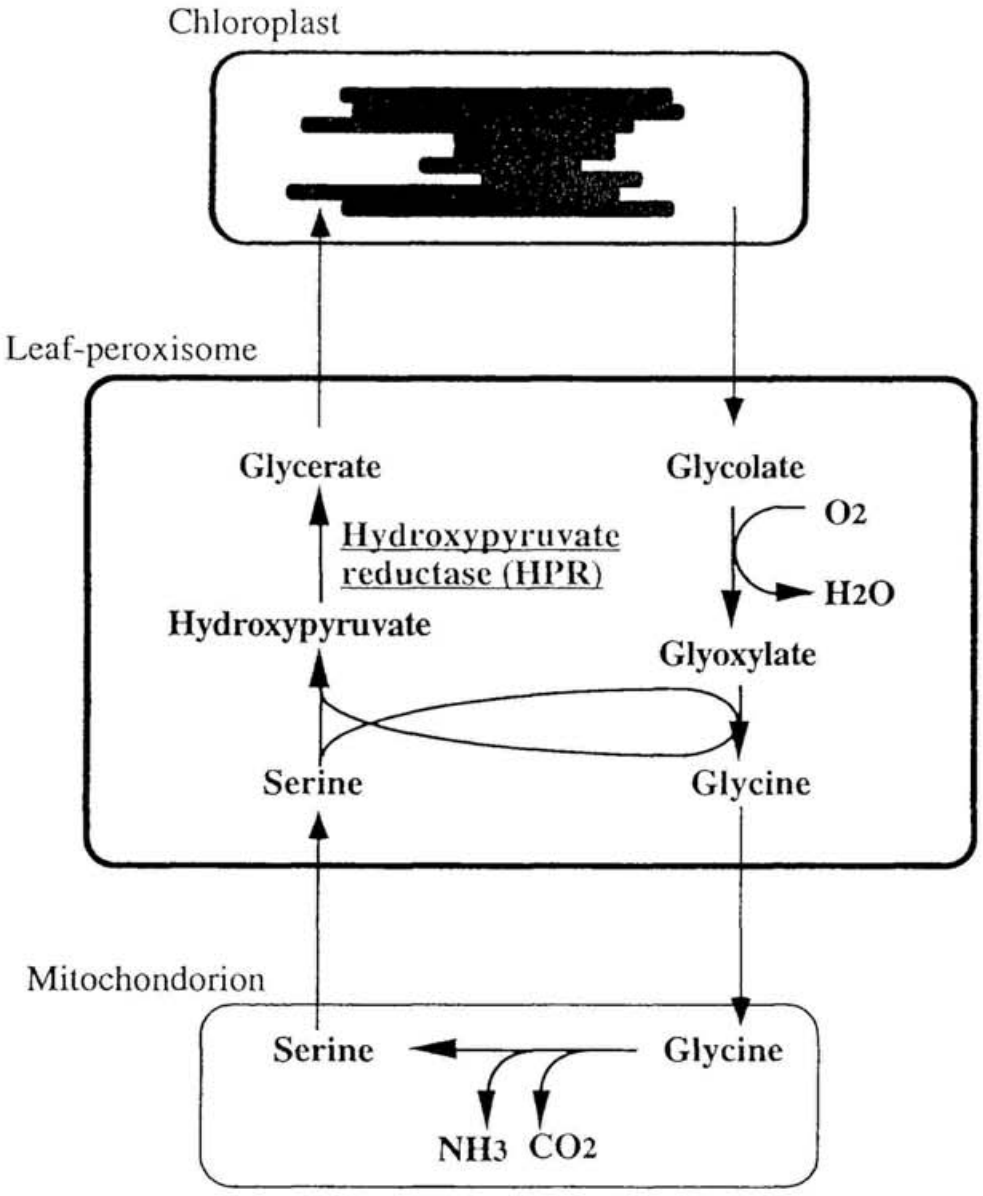


Figure 1-2. Pathway of photorespiration

1.2.2 Targeting signals into microbodies

Most microbody enzymes are transported into glyoxysomes, leaf peroxisomes or unspecialized microbodies due to the targeting signal located at the carboxy termini (PTS1) or at the amino terminal end (PTS2). As to the former, microbody enzymes are synthesized as mature forms with the tripeptide at the carboxy termini. Gould et al. (1987; 1988; 1989) reported that the unique tripeptide, Ser-Lys-Leu or their derivations, at the carboxy terminus could direct firefly luciferase to peroxisomes. It is assumed that these Ser-Lys-Leu-like sequences might be involved in the transport of microbody enzymes in eukaryotes since they function as PTS1 in insects, higher plants, yeast and protozoa (Gould et al., 1990; Blattner et al., 1992). In higher plants, Hayashi et al. (1997) demonstrated that the fusion proteins, which consists of β-glucuronidase and the Ser-Lys-Leu or their derivations at the carboxy terminus, are transported into glyoxysomes in etiolated tissues, leaf peroxisomes in greening tissues and unspecialized microbodies present in root tissues in transgenic *Arabidopsis*. It has been reported that some glyoxysomal and peroxisomal enzymes have PTS1 at their carboxy termini (Comai et al., 1989; Graham et al., 1989; Mori et al., 1991; Mano et al., 1996; 1997; Tsugeki et al., 1993). In yeasts and human, the putative PTS1 receptors, which are named PEX5, has been cloned (Leij et al., 1993; McCollum et al., 1993; Fransen et al., 1995; Wiemer et al., 1995). In contrast, PTS2, another targeting signal, is a conserved amino terminal nonapeptide (R/K)(L/V/I)(X)5(H/Q)(L/A) present in the presequence that is used by a smaller subset of microbody matrix proteins such as 3-ketoacyl-CoA thiolase, citrate synthase and malate dehydrogenase in higher plants (Kato et al., 1995; 1996; 1998). These proteins are synthesized as larger precursors and the presequence is cleaved after the import to microbodies. PEX7 is identified as the PTS2 receptor in yeast (Marzioch et al., 1994). Unfortunately, the component(s) including receptors for PTSs have not been identified in higher plants. The mechanism of targeting of some microbody enzymes such as catalase and PMP47 have not been clarified yet, since they do not contain typical PTS1 and PTS2 (Purdue and Lazarow, 1994), suggesting the presence of another targeting mechanism to microbodies.

1.3. Hydroxypyruvate reductase

NADH-hydroxypyruvate reductase (HPR; D-glycerate dehydrogenase, EC 1. 1. 1. 29) catalyzes the conversion of hydroxypyruvate to glycerate. This enzyme is known to be localized in leaf peroxisomes, which are specialized microbodies found in photosynthetic tissues in higher plants. As shown in Figure 1-2, it has a role in the glycolate pathway of photorespiration. Photorespiration involves the light-dependent uptake of O2 and release of CO2 during the metabolism of phosphoglycolate, the two-carbon product by the oxygenase activity of RubisCO. During photorespiration, up to 75% of the carbon diverted from the Calvin cycle as phosphoglycolate is returned to the cycle as 3-phosphoglycerate in a process involving metabolite flow through chloroplasts, mitochondria and leaf peroxisomes (Tolbert et al., 1968). HPR has been purified and characterized from *Chlamydomonas* (Husic and Tolbert, 1987) and spinach (Kleczkowski and Randall, 1988). This enzyme comprises two equal subunits and has a high affinity for hydroxypyruvate (K_m = 0.08 mM in the pH range 5.5-6.5). HPR can appreciably reduce glyoxylate of a reverse reaction but only at a high concentration (K_m = 20 mM), indicating that this is a side reaction of no physiological importance *in vivo*. HPR activity has been detected in mammals (Snell, 1986), bacteria (Kohn and Jakoby, 1968) and plants (Kleczkowski and Randall, 1988). cDNAs for HPR have been cloned from pumpkin (Hayashi et al., 1996), cucumber (Greenler et al., 1989), *Arabidopsis* (Mano et al., 1997) (Chapter 3) and methylotrophs (Chistoserdova and Lidstrom, 1991; Yoshida et al., 1994). With regard to gene, it has been cloned in only cucumber (Schantz et al., 1995), and *E.coli* also has *SerA* gene encoding phosphoglycerate dehydrogenase that catalyzes the oxidation of phosphoglycerate to phosphohydroxypyruvate. In cucumber, *cis*-elements necessary for light-regulation and leaf-specific expression have been determined using transgenic tobaccos that express β-glucuronidase under a series of deletions of HPR promoters. Interestingly, HPR activity is not associated with a common metabolic pathway in mammals, bacteria and plants. HPR in mammals catalyzes the second step in a pathway of serine degradation with the reaction product, D-glycerate, being channeled into the gluconeogenic pathway. In bacteria, it operates in a specialized

serine pathway in which one-carbon compounds, such as formaldehyde, is assimilated into cellular materials, whereas in higher plants, HPR functions in the photorespiratory glycolate pathway as mentioned above.

Previous work in our laboratory has reported that two kinds of cDNA clone for HPR (HPR1 and HPR2) were obtained from the cDNA library in pumpkin green cotyledons (Hayashi et al., 1996). The nucleotide sequences of these clones showed complete identity except for the region encoding the carboxy terminus. From the deduced amino acid sequences for HPR1 and HPR2, it was revealed that HPR1 protein, but not HPR2 protein, has the carboxy terminal tripeptide of Ser-Lys-Leu, which is known as a targeting signal to microbodies. In addition, the genomic structure around this region showed the presence of two pairs of GT-AG doublets within the intron (Figure 1-3). By the comparison of the genomic sequence with two cDNAs, it has been suggested that alternative splicing might give rise to two kinds of HPR mRNA. That is, HPR1 mRNA is produced after the longer intron is spliced out, whereas HPR2 mRNA is produced when the 17-bp intron sequence, which has a stop codon, is retained. As a result, HPR1 protein might be transported into leaf peroxisomes due to the targeting signal that is located at the carboxy terminus, and HPR2 protein might remain in the cytosol on account of the lack for such a targeting sequence. cDNA for cucumber HPR does not have this carboxy terminal tripeptide so it has been assumed as a novel targeting sequence in those days. However, the HPR gene of cucumber has the two pairs of GT-AG doublets at the similar position as the HPR gene of pumpkin, suggesting the possibility that cucumber HPR might be also produced by alternative splicing and that another HPR protein with the targeting signal into peroxisomes might exist in cucumber cells, although the cDNA has not been cloned (Hayashi et al., 1996). Nevertheless, no one has direct evidence whether alternative splicing is involved in the production of HPR in plants. If this speculation is correct, pumpkin HPR is the first example of microbody enzymes that is produced by alternative splicing and that has the possibility whose subcellular localizations are different. It has been reported that HPR activity and transcripts are both developmentally regulated and by light (Greenler et al., 1989), such as is glycolate

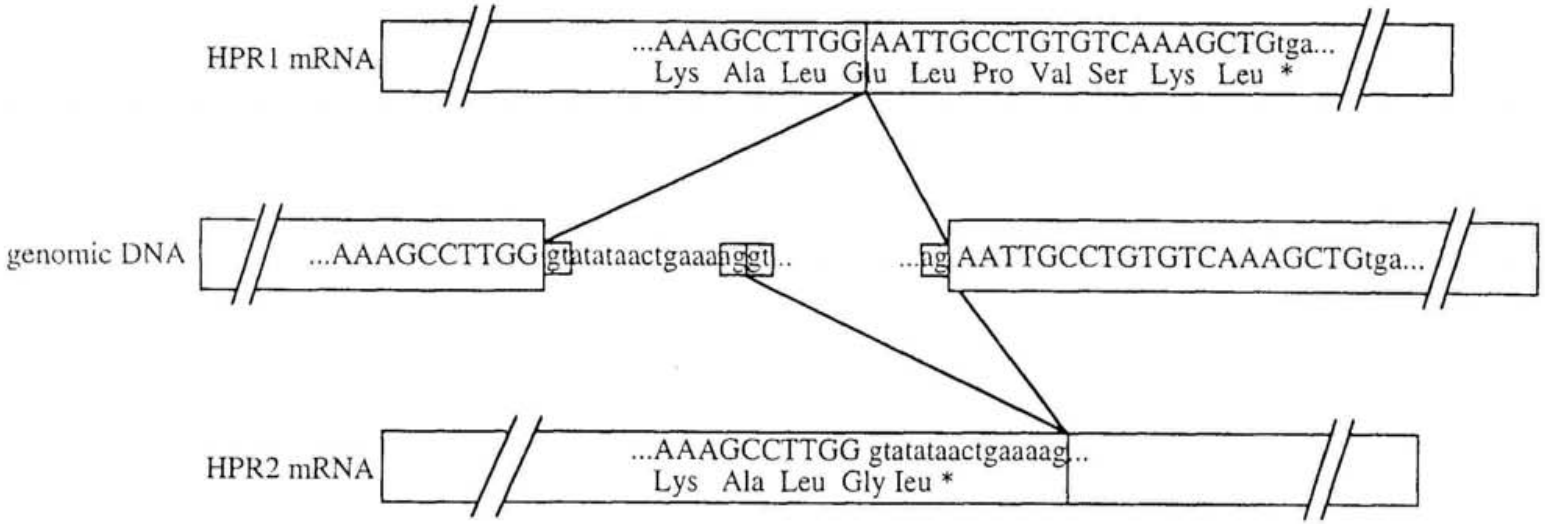


Figure 1-3. Schematic representation of mRNAs and the gene for pumpkin HPR

Uppercase letters and lowercase letters represent exons and introns, respectively. The deduced amino acid sequence is presented under the nucleotide sequence. Asterisks indicate the stop codons and boxes represent the consensus sequences found in the beginning (gt) and the end (ag) of the introns.

oxidase which is another leaf-peroxisomal enzymes (Tsugeki et al., 1993), and light promotes the microbody transition from glyoxysomes to leaf peroxisomes (Nishimura et al., 1986), indicating that this alternative splicing might be regulated by light.

1.4. Ascorbate peroxidase

Ascorbate peroxidase (APX), which uses ascorbate as the electron donor, has a key role in the removal of hydrogen peroxide. It contains one protoheme moiety per molecule and, based on its amino acid sequence, APX has been classified as a member of the Class I family of heme peroxidases where cyt *c* peroxidase is the core member of the family. In higher plants as shown in Figure 1-4, four isoenzymes of APX with different subcellular localizations have been reported, namely, microbody APX (mbAPX) (Yamaguchi et al., 1995; Bunkelmann and Trelease, 1996), cytosolic APX (cAPX) (Koshiba, 1993; Mittler and Zilinski, 1991), stromal APX (sAPX) (Chen and Asada, 1989; Ishikawa et al., 1996) and thylakoid-bound APX (tAPX) (Miyake et al., 1993; Ishikawa et al., 1996). Unlike classical plant peroxidase, APX has a remarkably high preference for ascorbate as a reductant. This enzyme has a higher activity when hydrogen peroxide concentration is low. APX activity is inhibited by thiol reagents, cyanide, azide, the primary products of oxidation of *p*-aminophenol, hydroxyurea and hydroxylamine. Of four isoenzymes, cAPX is localized in the cytosol of both photosynthetic and non-photosynthetic tissues. The function is still obscure, although it has been reported that mRNA for cAPX was induced by environmental stimulus such as drought and heat stress (Mittler and Zilinskas, 1992; 1994) and the rises of cAPX activity and the amount of cAPX mRNA during fruit ripening were reported in bell pepper (Schantz et al., 1995). mbAPX is localized on glyoxysomal and leaf peroxisomal membranes and plays a role in the reduction of hydrogen peroxide that is produced by the catalytic activities of β-oxidation in glyoxysomes and glycolate oxidase in leaf peroxisomes, and then leaks from glyoxysomes and leaf peroxisomes (Yamaguchi et al., 1995). Chloroplastic APXs, which are localized in the stroma (sAPX) and on the thylakoid membrane (tAPX),

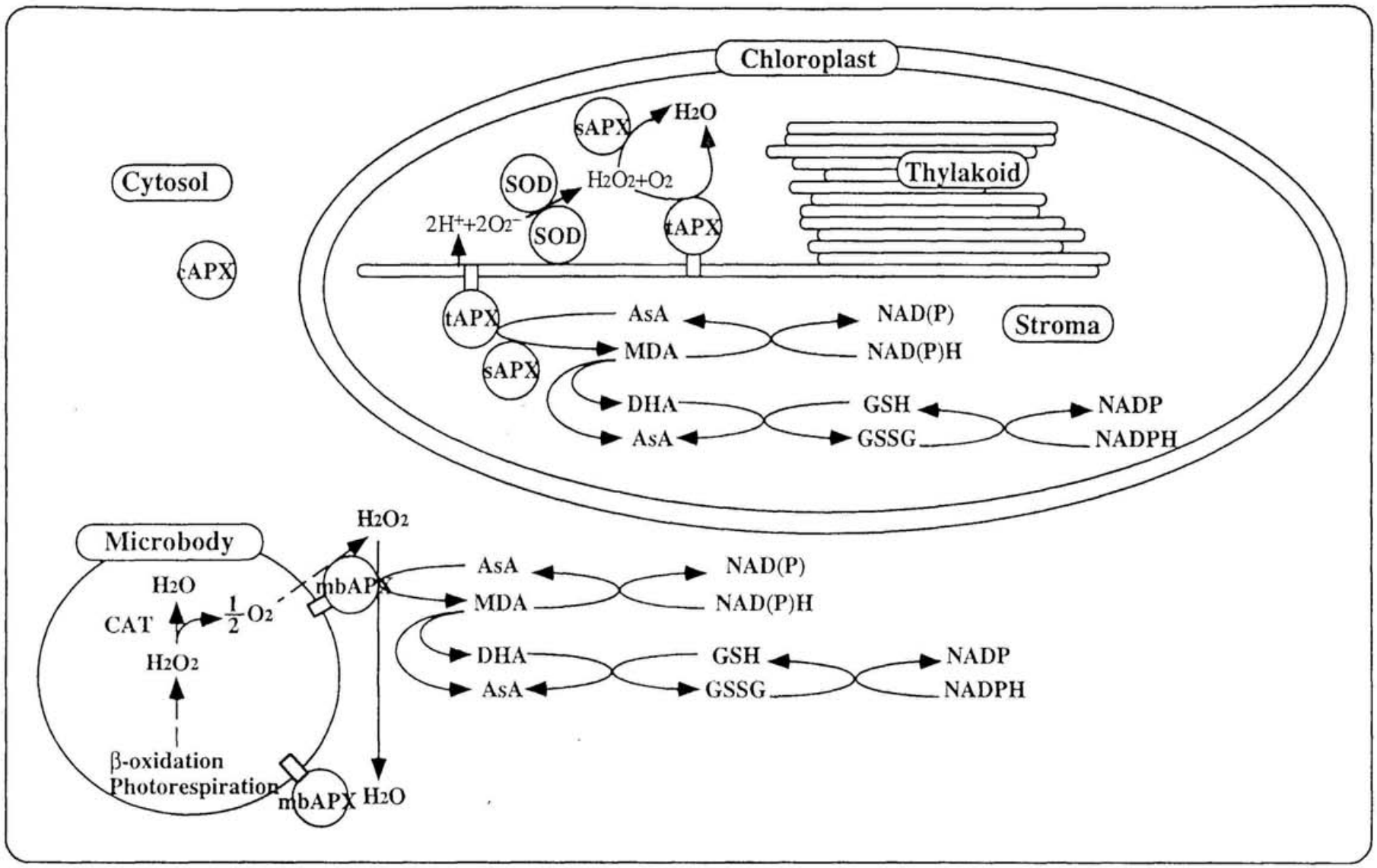


Figure 1-4. Possible functions of the four kinds of ascorbate peroxidase in plant cells

mbAPX, microbody APX; cAPX, cytosolic APX; sAPX, stromal APX; tAPX, thylakoid-bound APX; CAT, catalase; AsA, ascorbate; DHA, dehydroascorbate; SOD, superoxide dismutase

scavenge the hydrogen peroxide that is produced by the photochemical reactions in chloroplasts. Chloroplastic APXs have been purified, determined their partial amino acid sequences and characterized about their molecular properties well (Chen and Asada, 1989; Chen et al., 1992; Miyake et al., 1993). Miyake and Asada (1992) showed that the thylakoids had a nearly equal activity of APX to that in the stroma of spinach chloroplasts and that enzymatic properties of two chloroplastic APXs were very similar each other. Both sAPX and tAPX are inhibited by cyanide and azide, similar to cAPX, but they are very rapidly inactivated in the absence of ascorbate with compared to cAPX (Chen and Asada, 1989). Recently, cDNA for tAPX from pumpkin has been cloned and characterized in our laboratory (Yamaguchi et al., 1996) and Ishikawa et al. (1996) also isolated cDNAs for tAPX and sAPX from spinach leaves, showing that amino acid sequences of tAPX and sAPX were identical except for the deletion of the C-terminal domain of tAPX, although both APXs had a putative transit peptide into chloroplasts. To obtain further information on the regulation of gene expression of chloroplastic APX, I tried to isolate sAPX cDNA. Our data as shown in Chapter 4 demonstrated the presence of alternative splicing that is involved in the production of tAPX and sAPX mRNAs.

1.5. Aim of this study

Analyses on alternative splicing have been performed mainly in mammals and virus. However, recent studies revealed that alternative splicing is involved in the synthesis of some enzymes in higher plants, although the analysis gets behind to that in mammals. The two HPR proteins in pumpkin show complete identity except the carboxy domain that contains PTS1, indicating the involvement of alternative splicing, although direct evidence of this has not been obtained. Based on the function of HPR and microbody transition that is regulated by light, it is suggested that light might control this alternative splicing. Chapter 2 describes the presence of two HPR proteins with different subcellular localizations that is regulated by light. In higher plants, only cucumber HPR, except pumpkin HPR, has been registered (Greenler et al., 1989). Therefore, it is of

interest to examine another plants whether alternative splicing produces two kinds of HPR mRNA. The molecular cloning and characterization of *Arabidopsis* HPR was carried out as shown in Chapter 3. As another example of alternative splicing that is regulated by light and that is involved in the production of different subcellular localization, the molecular cloning and characterization of chloroplastic APXs are described in Chapter 4. These results show the novel alternative splicing that is controlled by light. The perspective of studies on alternative splicing in addition to composite discussion is described in Chapter 5.

Chapter 2

Alternative forms of pumpkin hydroxypyruvate reductase: identification and characterization

2.1. Summary

It has been speculated that alternative splicing appears to be involved in the production of two kinds of HPR mRNA although direct evidence of this has not been obtained. To investigate the presence of two HPR proteins, immunochemical analysis was carried out. Application of an HPR-specific antibody resulted in the detection of two electrophoretically similar polypeptides in total extracts from pumpkin cotyledons and provides evidence that their expression is regulated developmentally and by light. Subcellular fractionation of pumpkin cells showed that the activity of HPR is detected in the cytosolic and leaf peroxisomal fractions. Moreover, indirect fluorescent microscopy and analysis of transgenic tobacco cultured cells (BY-2) and *Arabidopsis* using green fluorescent protein (GFP) gave fluorescent patterns with different subcellular localizations (peroxisomes and the cytosol). These results confirmed the existence of two HPR proteins that were localized in different parts of pumpkin cells, indicating the existence of a novel alternative splicing that is regulated developmentally and by light, and that gives rise to different subcellular localizations in pumpkin cells.

2.2 Introduction

As mentioned in Chapter 1, the two kinds of cDNA for pumpkin HPR (HPR1 and HPR2) have been cloned in our laboratory. Deduced amino acid sequences and the structure of genomic DNA (Figure 1-3) indicated that the two HPR proteins were produced by alternative splicing from a single gene and that HPR1 and HPR2 proteins were localized in leaf peroxisomes and the cytosol, respectively, because HPR1 protein, but not HPR2 protein, has a targeting signal at the carboxy terminus. However, direct evidence about the presence of two HPR proteins in different parts of pumpkin cells has not been obtained. It was therefore necessary to detect two proteins and mRNAs corresponding to them. The metabolic role of HPR2 in the cytosol has not been identified yet. Our findings indicate the presence of a novel interaction such as the exchange of substrates between leaf peroxisomes and the cytosol or that HPR2 might have an

unidentified role in the cytosol. In mammals, alternative splicing sometimes shows the tissue or developmental stage specificities. In higher plants, some reports revealed the presence of alternative splicing dependent on the tissue specificity (Görlach et al., 1995; Lumbreras et al., 1995; Tamaoki et al., 1995; Thorbjørnsen et al., 1996; Tobias and Nasrallah, 1996; Macknight et al., 1997). It is therefore interesting to investigate whether alternative splicing in pumpkin HPR shows the tissue or developmental specific fashions. Moreover, as light regulates gene expression for HPR (Greenler et al., 1989) and promotes the microbody transition from glyoxysomes to leaf peroxisomes (Nishimura et al., 1986), it was anticipated that light might take part in the mechanism of alternative splicing for pumpkin HPR. It has not been reported on the mechanism of alternative splicing that is regulated by environmental factors such as light. Pumpkin HPR is also the first example of microbody enzymes that is produced by alternative splicing and whose localizations within cells are different.

2.3. Materials and methods

Plant material

Pumpkin (*Cucurbita* sp. cv. Kurokawa Amakuri Nankin) seeds were soaked overnight and germinated in moist rock fiber (66R; Nitto Bouseki, Chiba, Japan) at 25°C or 35°C in darkness. Some of the seedlings were transferred to continuous light at the 5-day stage. Suspension-cultured tobacco cells (*Nicotiana tabacum* L. cv Bright Yellow 2), kindly provided by Dr. K. Nakamura (Nagoya University), were maintained on an orbital shaker at 26.5°C in the dark. Cells were subcultured once a week using Murashige-Skoog medium.

Plasmid constructions and transformation into BY-2 and *Arabidopsis thaliana*

The constructs which were used to transform BY-2 and *Arabidopsis thaliana* were based on sGFP-TYG that was kindly provided by Dr. Y. Niwa (University of Shizuoka).

pSGFP-BE, which had a *Bam*H I site in the 5' franking region and *Bgl* II and *Eco*R I sites on either side of the stop codon of *sGFP*, was generated by PCR using primers GFPsF (5'-CCGGATCCATGGTGAGCAAGGGCGAGGAG-3') and GFPsR (5'-GGGAATTCTCAGAGATCTCCCTTGTACAGCTCGTCCAT-3'). The fragment was subcloned into a T-vector prepared using pBluescript KS$^+$ as described previously (Marchuk et al., 1990). In order to make the constructs for pMAT-SGFP-H1 and pMAT-SGFP-H2, the *Bgl* II-*Eco*R I fragment of the DNA fragment in pSGFP-BE was replaced with two kinds of double-stranded DNA produced by the annealing of complementary oligonucleotides. The complementary oligonucleotides were designed to produce protruding ends that ligate to the *Bgl* II and *Eco*R I sites of the *sGFP-BE* gene. The nucleotide sequences of the coding strands of the synthetic oligonucleotides used to construct chimeric genes are shown in Figure 2-7. The *Bam*H I-*Eco*R I fragments that contain chimeric genes were inserted into the *Bgl* II-*Eco*R I site of a Ti-plasmid, pMAT037 (Matsuoka and Nakamura, 1991). The Ti-plasmids produced were then transformed into *Agrobacterium tumefaciens* (strain EHA101) by electroporation. Transformation of BY-2 using *Agrobacterium tumefaciens* was performed according to the method of Matsuoka and Nakamura (1991). Transformation of *Arabidopsis thaliana* (ecotype Columbia) was carried out by the infiltration method (Bechtold et al., 1993). Primary transformants were designated T0 plants. T1 seeds collected from T0 plants were surface sterilized in 2% NaClO, 0.02% Triton X-100 and grown on germination media (2.3 mg ml^{-1} MS salt (Wako, Osaka, Japan), 1% sucrose, 100 μg ml^{-1} myo-inositol, 1 μg ml^{-1} thiamin-HCl, 0.5 μg ml^{-1} pyridoxine, 0.5 μg ml^{-1} nicotinic acid, 0.5 mg ml^{-1} MES-KOH (pH 5.7), 0.2% gellan gum (Wako, Osaka, Japan)) containing 100 μg ml^{-1} of kanamycin. T2 seeds were collected from approximately 10 independent T1 plants. T2 plants were selected based on their fluorescence observed with fluorescent microscopy (see below).

Northern blot analysis

Ten μg of total RNA extracted from pumpkin cotyledons grown under various stages was fractionated on a 1% gel that contained 0.66 M formaldehyde and 10 mM MOPS (pH 7.0). RNA was transferred to a Zeta-Probe blotting membrane (BIO-RAD, CA, USA) by the capillary method and fixed by exposure to UV-light (Funa-UV-Linker, model FS-800; Funakoshi, Tokyo, Japan). The fragment corresponding to HPR cDNA was labeled with [^{32}P]-dCTP (Amersham Japan) using a Megaprime™ DNA labeling system (Amersham, Tokyo, Japan). The membrane was hybridized in 50% formamide, 0.12 M sodium phosphate (pH 7.2), 0.25 M sodium chloride, 7% SDS and 1mM EDTA (pH 8.0) with 1.0×10^6 cpm·ml^{-1} of radiolabeled DNA probe for 18 h at 42°C. The membrane was washed at 42°C in 2 × SSC plus 0.1% SDS for 15 min, in 0.2 × SSC plus 0.1% SDS for 15 min, in 0.1 × SSC plus 0.1% SDS for 15 min. The membrane was used to expose X-ray film and radioactivity was measured on the imaging plate of a Bio-imaging analyzer (FUJIX BAS2000; FUJI Photo Film, Tokyo, Japan).

Immunoblot analysis

Pumpkin cotyledons grown under various conditions and various tissues were homogenized with the extraction buffer (100mM Tris-HCl, pH 6.8, 1mM EDTA, pH 8.0, 0.1% Triton X-100 and 1mM PMSF) and then the homogenates were centrifuged at 15,000 × *g* for 25 min. The protein content of each extract was estimated using a protein assay kit (Nippon Bio-Rad Laboratories, Tokyo, Japan) with bovine gamma albumin as a standard protein. Total proteins were separated by SDS-PAGE on a 7.5% polyacrylamide gel as described by Laemmli (1970) and transferred to a nylon membrane (Schleicher & Schuell, Dassel, Germany) in a semidry electroblotting system. Immunologic reactions were detected by monitoring the activity of horseradish peroxidase antibodies against rabbit IgG (ECL system; Amersham, Japan).

Southern blot analysis

Total DNA (5 μg) was digested with *Bam*H I, *Eco*R V, *Hin*c II, *Sma* I and *Sph* I. The products were fractionated on a 0.8% agarose gel, transferred to a Zeta-Probe

blotting membrane (BIO-RAD, CA, USA) by the capillary method and fixed by exposure to UV-light (Funa-UV-Linker, model FS-800; Funakoshi, Tokyo, Japan). The fragment corresponding to HPR cDNA was labeled with [^{32}P]-dCTP (Amersham Japan) using a Megaprime^{TM} DNA labeling system (Amersham, Tokyo, Japan). The conditions of hybridization and washing of the membrane were the same as previously described in the section of Northern blot analysis. The membrane was used to expose X-ray film.

RT-PCR analysis

First-strand cDNA was generated using a Ready-To-Go T-primed first-strand kit (Pharmacia Biotech, Tokyo, Japan) from poly(A)$^+$ RNA templates prepared from 5-day dark-grown or 5-day dark- and 2-day light-grown cotyledons using Oligotex-dT 30 <Super> (Roche Japan, Tokyo, Japan). PCR was performed to amplify each HPR mRNA. The reaction mixture contained 1 unit of EX Taq DNA polymerase (Takara Shuzo, Kyoto, Japan), an upstream primer (N4; 5'-GCCGCTCTTAATGTTCTGGGA-3'), a downstream primer (B2C; 5'-TAATGCTTGCGATTGGTGCTG-3') and an appropriate buffer in a total volume of 50 µl. Each cycle was at 94 °C for 45 sec and 64 °C for 45 sec.

Subcellular fractionation

A 2 g of 5-day dark- and 2-day light-grown pumpkin cotyledons was homogenized with razor blades for 5 min in 6 ml of chopping buffer (10mM Tricine-HCl, pH 7.5, 1mM EDTA and 0.5M sucrose). The homogenate was passed through four layers of cheesecloth. Then 1.5 ml of filtered sample was layered onto a sucrose gradient that consisted of a 1 ml cushion of 60% (w/w) sucrose and 11 ml of a linear sucrose gradient and was centrifuged at 25,000×*g* for 3 h in an SW 28-1 rotor in an ultracentrifuge (model XL-90; Beckman, Fullerton, CA, USA). After centrifugation, fractions (0.5 ml each) were collected with an automatic liquid charger (ALC-2L; Advantec, Tokyo, Japan). All procedures were carried out at 4°C. Assays of HPR and

catalase were carried out according to De Bellis and Nishimura (1991) and Yamaguchi and Nishimura (1984).

Immunofluorescent staining of thin sections of pumpkin leaves

Pumpkin leaves were cut into 1-mm cubes and vacuum-infiltrated in a fixative (4% formaldehyde, 50mM cacodylate buffer, pH 7.4, 60mM sucrose 0.001% Triton X-100). Fixed leaves were then incubated in the same fixative for a further 2 h at 20°C. Then the cubes were embedded in 5% agar. Thin sections (30 μm) were cut with razor blades on a Microslicer DTK-1000 (Dosaka, Japan). The sections were fixed on glass slides and treated with 0.1% pectolyase Y-23 for 2 h at 30°C. Slides were washed three times with excess PBS for 5 min each. The sections were further incubated with 0.3% Triton X-100 for 15 min at 20°C. Slides were washed three times with excess PBS for 5 min each and were sections treated with 5% BSA in PBS for 1 h at 20°C. Then the sections were incubated with the first antibody for 1 h at 37°C, washed three times with excess PBS for 5 min each and treated with 5% BSA in PBS for 30 min at 20°C. The sections were incubated with 1:100 diluted FITC-conjugated goat antibodies against rabbit IgG and then washed three times with excess PBS for 5 min each.

Fluorescent microscopy

The photographs of fluorescence of the immunostained samples from pumpkin leaves and transgenic suspension-cultured tobacco cells were taken using an epifluorescence microscope (BHS2; Olympus, Tokyo, Japan) with a filter set (80P000 PINKEL 602, Photometrics, Japan) and Fujichrome 1600 film (Fuji Film, Tokyo, Japan).

Confocal microscopy

The immunostained leaves of pumpkin and transgenic *Arabidopsis thaliana* were mounted in water under glass coverslips. The specimens were examined using a LSM-GB200 laser-scanning confocal microscope equipped with a krypton-argon laser and a

filter set comprising BP535 and DM488 (Olympus, Tokyo, Japan) or using a TCS NT laser-scanning confocal microscope with a FITC filter set (Leica, Tokyo, Japan). Photographs were taken using Fujichrome 100 film (Fuji Film, Tokyo, Japan).

2.4. Results

Pumpkin HPR is encoded by a single copy gene

To investigate the presence of another HPR gene, a Southern blot analysis was carried out. Genomic DNA from pumpkin cotyledons was isolated, digested with *Bam*H I, *Eco*R V, *Hin*c II, *Sma* I and *Sph* I and subjected to a Southern blot hybridization using pumpkin HPR cDNA as a probe. As shown in Figure 2-1, HPR cDNA hybridized to single fragments when digested with *Bam*H I and *Sma* I, while it gave three, four or two bands when digested with *Eco*R V, *Hin*c II and *Sph* I, respectively. The HPR gene was cloned and the sites for restriction enzymes in the HPR gene was checked by digestion with above restriction enzymes. At a result, there were two sites for *Eco*R V, three sites for *Hin*c II and one site for *Sph* I (data not shown). The bands shown in Figure 2-1 seemed to have been produced by internal sites for each restriction enzyme in the HPR gene. In addition, only one kind of genomic sequence was obtained when the genomic DNA was amplified using specific primers (Hayashi et al., 1996), indicating that pumpkin HPR exists as a single-copy gene.

Developmental changes in the levels of HPR mRNA

The change in HPR mRNA from various developmental stages was investigated by a Northern analysis as shown in Figure 2-2. The relative amounts of HPR mRNA were determined densitometrically after a Northern blotting. The amount of HPR mRNA was low until the 3-day stage and then increased gradually. When 5-day-old seedlings were exposed to continuous light, a dramatic increase in the level of mRNA was observed (open circles in Figure 2-2). However, when the cotyledons were kept in darkness, the amount of mRNA reached the maximum level at 5 days after germination and then

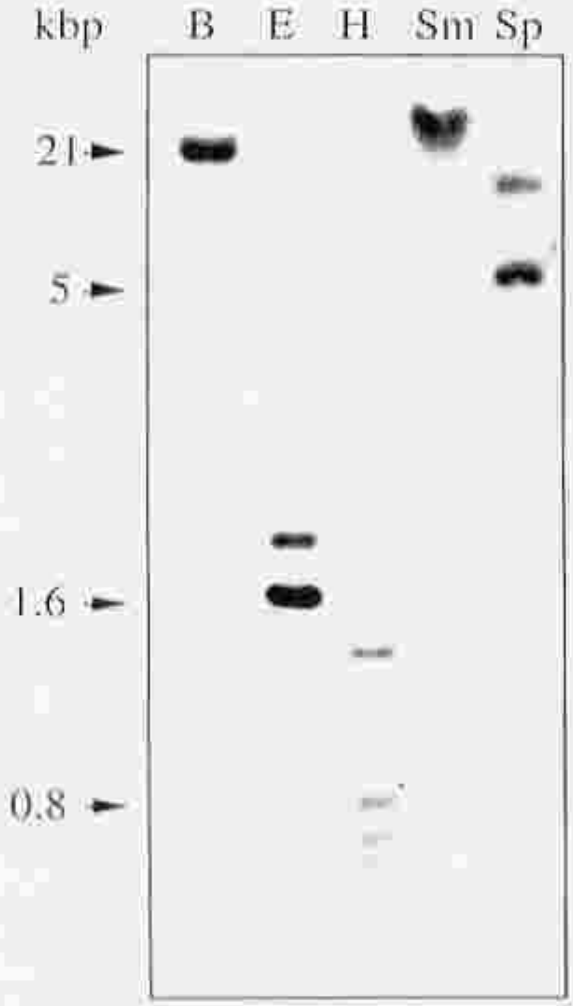


Figure 2-1. Southern blot analysis of pumpkin genomic DNA.

Genomic DNA (5 μg each) was digested with the restriction endonucleases indicated, separated on a 0.8% agarose gel, transferred to a Zeta-Probe blotting membrane (BIO-RAD) and probed with a [^{32}P]-labeled pumpkin HPR cDNA fragment. The size of DNA fragments are shown in kbp on the left. B, *Bam*H I; E, *EcoR* V; H, *Hinc* II; Sm, *Sma* I; Sp, *Sph* I.

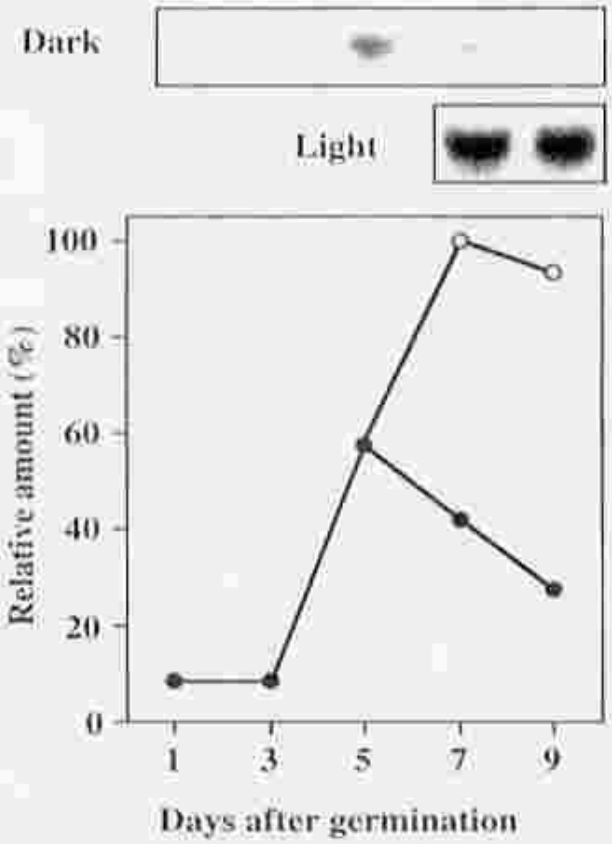


Figure 2-2. Developmental changes in levels of HPR mRNA in pumpkin cotyledons after germination.

Ten μg of total RNA extracted from pumpkin cotyledons grown under various conditions was separated on a 1% agarose gel. The RNA transferred to a Zeta-Probe membrane (BIO-RAD) was hybridized with a [^{32}P]-labeled cDNA fragment for pumpkin HPR. Radioactivity was measured densitometrically. (●) seedlings grown in darkness; (○) seedlings exposed to continuous illumination at the 5-day stage.

dropped rapidly (closed circles in Figure 2-2). Similar results have been previously reported for another peroxisomal enzyme, glycolate oxidase (Tsugeki et al., 1993), indicating that the regulation of gene expression for HPR is controlled developmentally and by light, as is the expression of other peroxisomal enzymes.

Light controls alternative splicing for HPR

A Northern blot hybridization is unable to resolve the HPR1 and HPR2 mRNAs due to their similar lengths. In order to solve this problem, an RT-PCR analysis was performed to examine whether light was related to alternative splicing of HPR pre-mRNA. As templates, first-strand cDNAs were synthesized from poly(A)$^+$RNAs prepared from 5-day dark-grown and 5-day dark- and 2-day light-grown cotyledons. Figure 2-3 shows the result of one of several identical experiments. The combination of N4 and B2C primers is able to hybridize to both HPR1 and HPR2 cDNAs. When each plasmid was used as a template, different truncated fragments were amplified (lane 1 and 2 in Figure 2-3). Low amounts of two bands, which corresponded to HPR1 and HPR2 fragments, were detected at the 5-day-old stage (lane 3 in Figure 2-3). In this case, the amounts were almost the same. On the contrary, exposure to continuous light for 2 days enhanced the accumulation of HPR2 mRNA more than that of HPR1 mRNA (lane 4 in Figure 2-4), suggesting that light might shift the pattern for splicing of HPR pre-mRNA to greater production of HPR2 mRNA.

Two kinds of polypeptide are detected during development after germination

To identify the presence of two HPR proteins and investigate the developmental changes after germination, an immunoblot analysis was carried out using total extracts from pumpkin cotyledons under various developmental stages. Figure 2-4 shows that two electrophoretically similar polypeptides with molecular masses of about 42 kDa were recognized in the total extracts prepared from 7-day- and 9-day-old cotyledons by antibodies against spinach HPR. This is in good agreement with the molecular masses

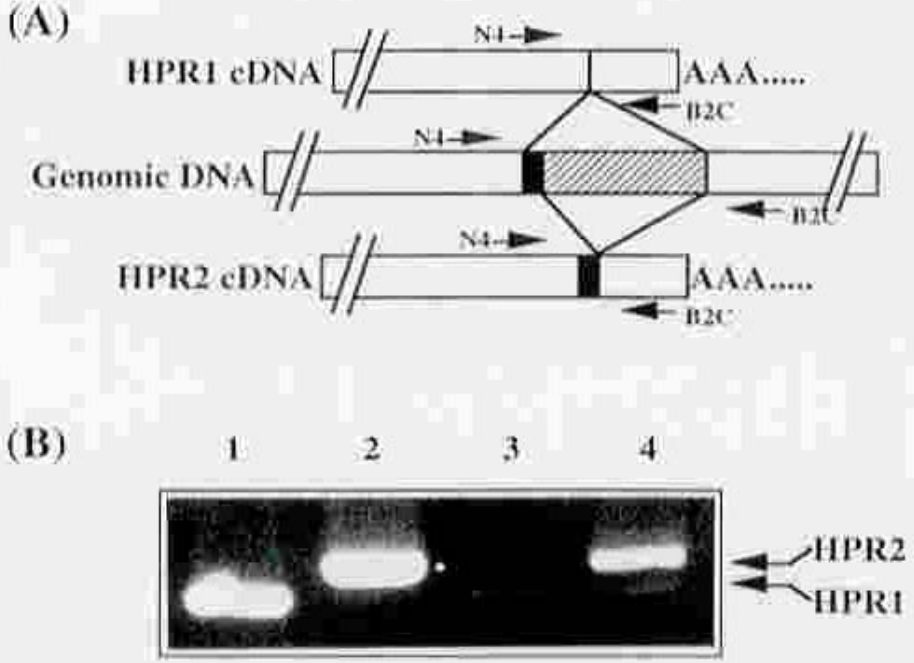


Figure 2-3. RT-PCR analysis

(A) Schematic representation of cDNAs and the gene for pumpkin HPR The hatched and filled boxes correspond to the two parts of a doublet of intron. The filled box indicates a 17-bp intron that is inserted into HPR2 cDNA. Primers for RT-PCR, N4 and B2C, are shown with arrowheads indicating the polarity. (B) RT-PCR products for truncated HPR1 and HPR2 detected on an agarose (1%)/NuSieve (3%) gel stained with ethidium bromide. Lanes were loaded with amplified cDNA derived from HPR1 cDNA (lane 1), HPR2 cDNA (lane 2), first strand cDNA prepared from 5-day dark-grown pumpkin cotyledons (lane 3) and first strand cDNA prepared from 5-day dark- and 2-day light-grown pumpkin cotyledons (lane 4).

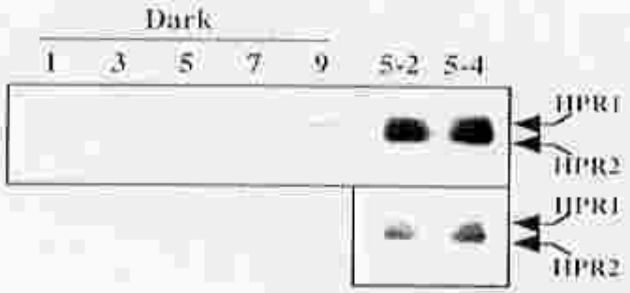


Figure 2-4. Developmental changes of HPR1 and HPR2 polypeptides.

Total protein prepared from cotyledons grown under various conditions was subjected to SDS-PAGE (10μg each), which was followed by immunodetection with antibodies against HPR. HPR1 and HPR2 polypeptides are indicated by arrows, respectively. Lower photograph represents the immunodetection under shorter exposure time. Numbers above photographs represent the days after germination. 5-2 and 5-4 indicate that cotyledons that were grown in the dark for 5 days and then kept in continuous light for 2 days or 4 days from the 5-day stage.

calculated from the deduced amino acids. Judging from the molecular masses of the HPR1 (42,305 Da) and HPR2 (41,709 Da) proteins, upper and lower polypeptides must correspond to HPR1 and HPR2 proteins, respectively. With respect to the developmental changes of these polypeptides, the amounts of both polypeptides increased gradually during germination in darkness. When seedlings were transferred to continuous light at the 5-day stage, the abundances of the two polypeptides were increased markedly (lane 5-2 and 5-4 in Figure 2-4). A difference between the accumulation of both polypeptides and that of mRNA was observed after 5 days. When seedlings were grown in darkness, the amount of mRNA dropped after 5 days (Figure 2-2), whereas those of polypeptides continued to increase, suggesting that the amounts of HPR proteins are regulated post-transcriptionally. Moreover, from this result, the ratio of HPR1 to HPR2 was estimated to be 1.0 when total extracts prepared from dark-grown cotyledons was used. However, the ratio of HPR1 to HPR2 was changed from 1.0 to 2.4 when the seedlings were transferred to continuous light after 5 days, implying that this alternative splicing is regulated by light.

Subcellular localization of pumpkin HPR

If HPRs exist in the cytosol and leaf peroxisomes, HPR activity is to be detected in the cytosolic and peroxisomal fractions after subcellular fractionation of pumpkin cells. In order to investigate this postulation, subcellular fractionation of pumpkin cotyledons by sucrose density gradient centrifugation was carried out. Odd fractions were subjected to an immunoblotting analysis and the estimation of HPR activity. Catalase activity was also estimated as a leaf peroxisomal marker enzyme. As shown in Figure 2-5, catalase activity was detected in the supernatant fractions that contain cytosolic enzymes (fraction nos. 1 and 3) as well as in the leaf-peroxisomal fraction (fraction no. 23), since leaf peroxisomes are so fragile that enzymes in the leaf peroxisomal matrix are released. The scales in Figure 2-5 were adjusted so that the activities of HPR and catalase in the peroxisomal fraction (no. 23) are the same. If the percent of HPR that leaks from leaf peroxisomes is the same as that of catalase, and if HPR, like catalase, is exclusively

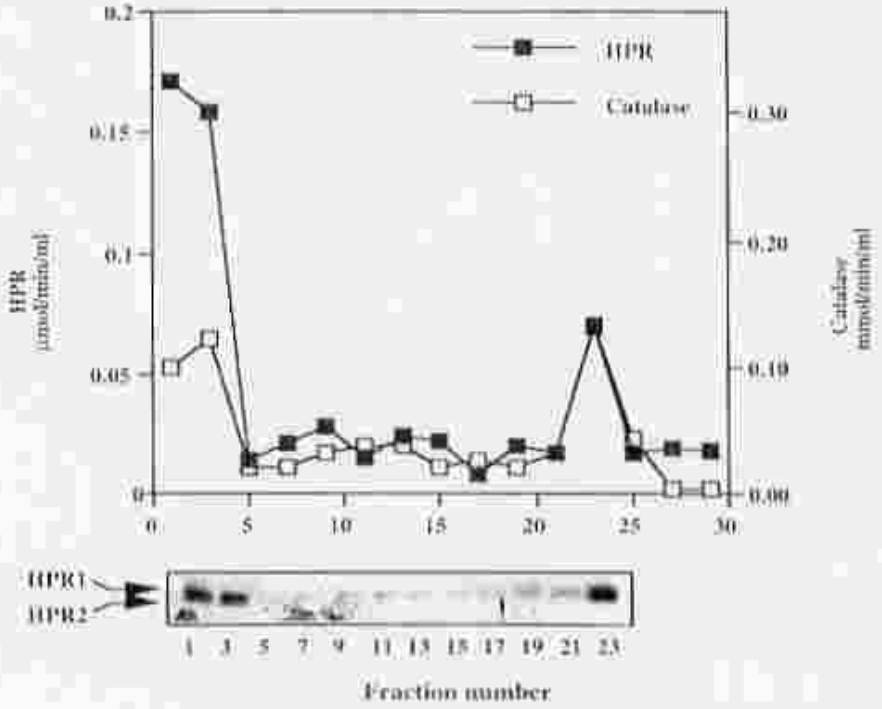


Figure 2-5. Subcellular distribution of HPR in pumpkin cotyledons.

Total protein prepared from 5-day dark- and 2-day light grown pumpkin cotyledons was homogenized and the homogenate was subjected to sucrose density gradient centrifugation. After fractionation, activities of HPR and catalase were measured. HPR was detected immunologically with antibodies against spinach HPR. Upper panel; activities of HPR (■) and catalase (□). Lower panel; 2% (v/v) of each fraction was subjected to SDS-PAGE (7.5% acrylamide). Arrows on the left represent the HPR1 and HPR2 polypeptides.

localized in leaf peroxisomes, the activities of HPR and catalase in the supernatant fraction should be similar. However, high activity of HPR was detected in the cytosolic fraction compared to that of catalase. It is speculated that this high activity originated from HPR2 in the cytosol in addition to the HPR1 that leaked from leaf peroxisomes. Antibodies against HPR certainly recognized the two polypeptides in the cytosolic fractions and one polypeptide in the peroxisomal fraction (lower panel in Figure 2-5). Moreover, indirect immunofluorescent analysis was performed to confirm the presence of two HPR proteins in pumpkin cells. Cells isolated from pumpkin leaves were allowed to react with the antibodies against HPR or catalase, and the immunoreaction was visualized with FITC-conjugated second antibodies. As shown in Figure 2-6, leaf peroxisomes are the only part in which well defined fluorescence was observed with the FITC filter after staining with catalase-specific antibodies (A and B in Figure 2-6). Application of antibodies against HPR, however, revealed the weak fluorescence throughout the entire cells in addition to strong spherical signals (C and D in Figure 2-6). The weak fluorescence observed over the entire cell indicated the presence of cytosolic HPR, concluding the existence of two kinds of HPR proteins within pumpkin cells. One is in leaf peroxisome and the other is in the cytosol.

Analysis of organelle targeting within transgenic BY-2 cells and root tissues of *Arabidopsis*

In order to confirm the subcellular localizations of the two HPR proteins, chimeric genes for fusion proteins containing green fluorescent protein (GFP) was constructed. As mentioned previously, the amino acid sequences of HPR1 and HPR2 differ only in the carboxy terminal region. Therefore, two chimeric proteins consisting of GFP and the carboxy terminal ten residues of either HPR1 or HPR2 were constructed (Figure 2-7). Plasmid constructs were introduced into BY-2 cells (Figure 2-8) and *Arabidopsis* (Figure 2-9) via *Agrobacterium*. GFP without a targeting sequence tends to be localized in the cytosol and the nucleus (G and H in Figure 2-8 and B and D in Figure 2-9). In the case of the chimeric protein with HPR1, well defined spots were observed to be distributed

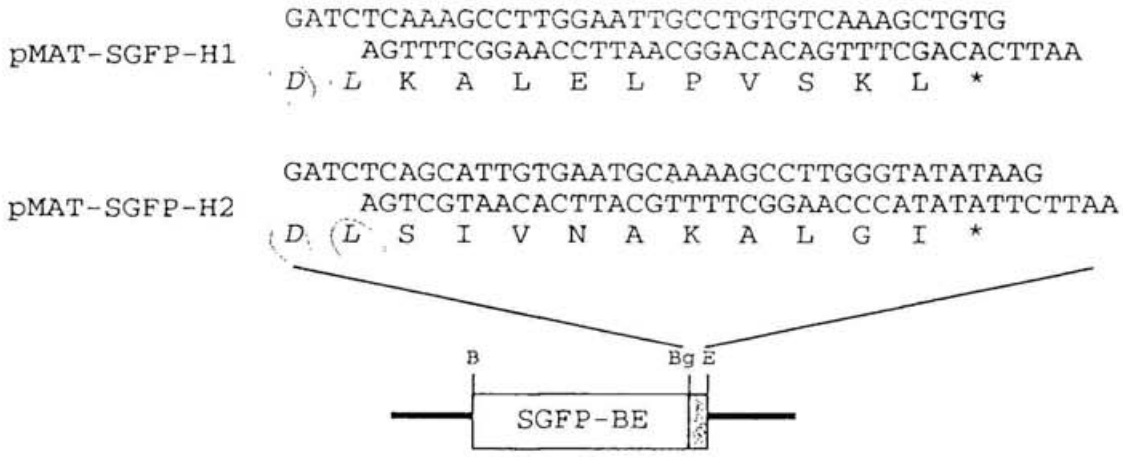


Figure 2-7. Structures of chimeric constructs used for making transgenic plants.

The nucleotide sequences of DNA fragments inserted into the *Bgl* II-*Eco*R I site of pMAT-SGFP-BE are shown. The amino acid sequences are presented under the nucleotide sequences. The two amino acids shown in italics act as a linker between sGFP and each polypeptide. B, *Bam*H I; Bg, *Bgl* II; E, *Eco*R I

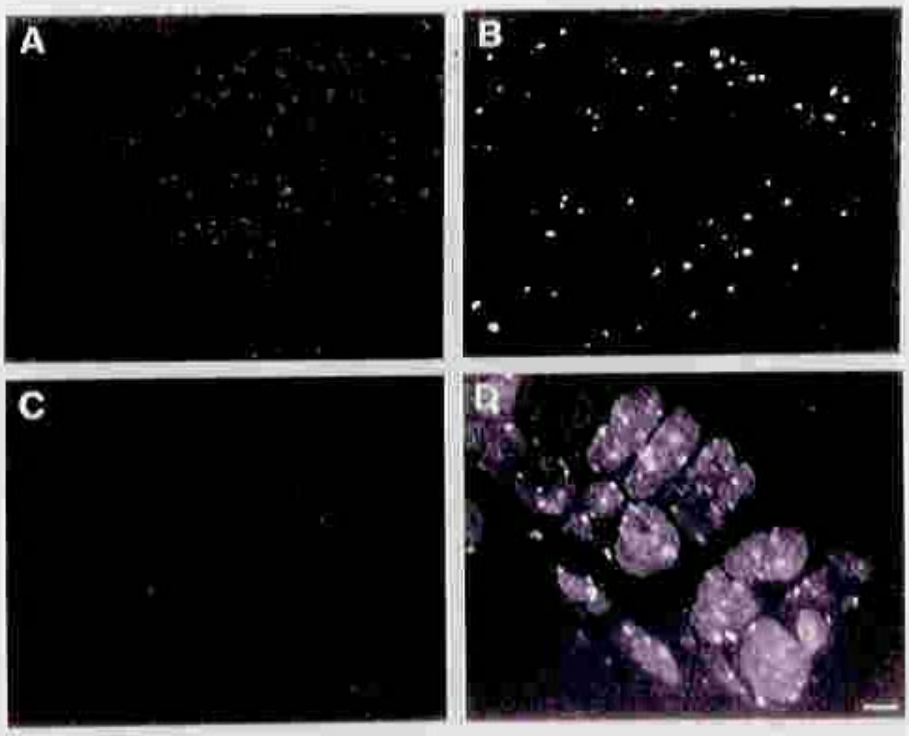


Figure 2-6 Microscopic observations of pumpkin leaves stained with FITC.

(A) and (B), Immunofluorescent and confocal images, respectively, of thin sections of pumpkin leaf stained with catalase antibody and then with FITC-conjugated second antibody. (C) and (D), Similar images except sections were first stained with HPR antibody. Bar indicates 10 μm.

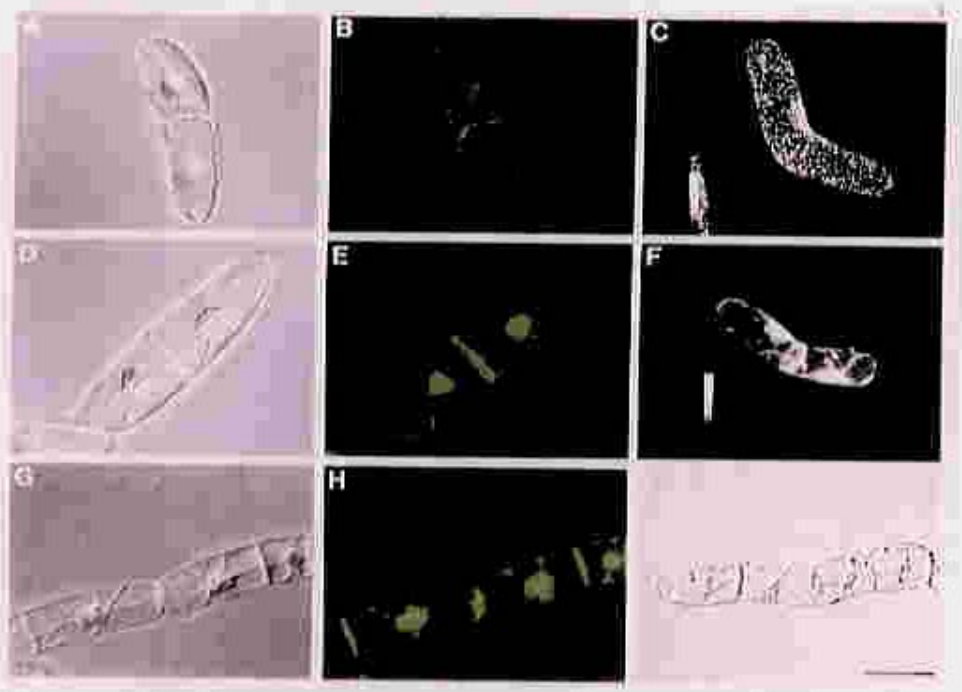


Figure 2-8. Subcellular localizations of transgenic tobacco BY-2 cells.

Wild typed cells (I) and transgenic cells (A-H) were observed in light field (Nomarski optics; A, D, G and H) and with blue light excitation (470-490 nm; B, E and H). (C) and (F) represent 3-D structures. (A), (B) and (C), pMAT-SGFP-H1; (D), (E) and (F); pMAT-SGFP-H2; (G) and (H); pMAT-SGFP-BE. Bar indicates 10 μm.

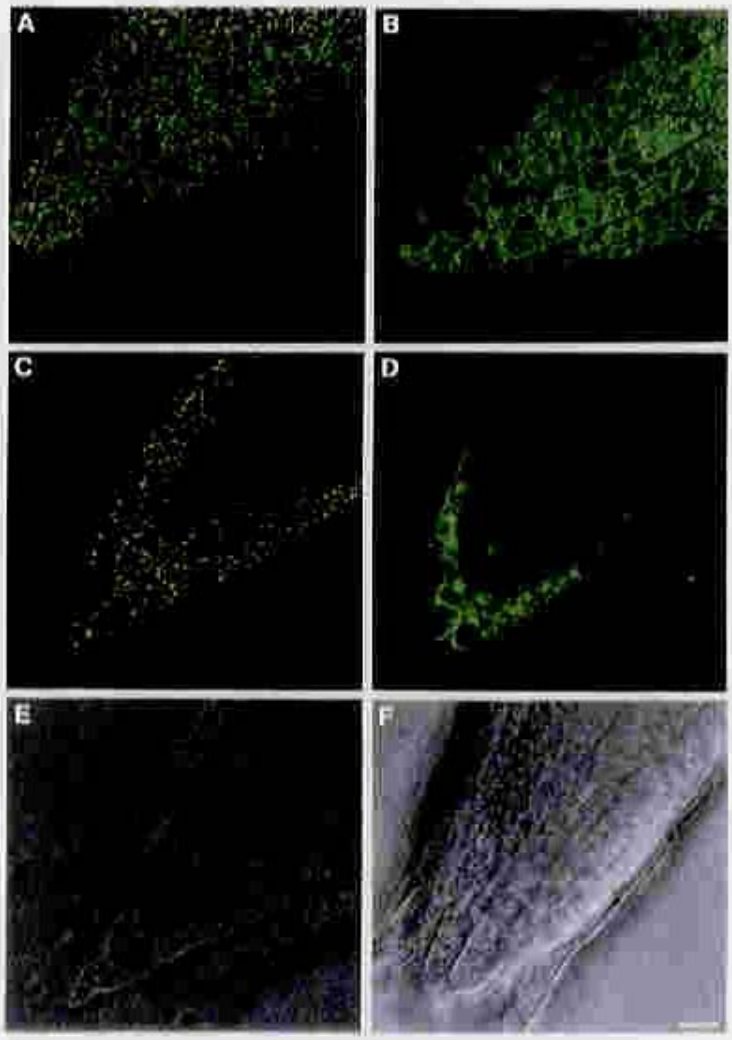


Figure 2-9. Subcellular localizations in root cells of transgenic *Arabidopsis thaliana*. (A) and (B), 3-D images of SGFP-H1 and SGFP-BE in root cells, respectively. (C) and (D), The same samples viewed with a confocal microscope. (E) and (F), The same samples viewed with a light microscope. Bar indicates 10 μm.

diffusely throughout the cytosol in BY-2 cells (B and C in Figure 2-8) and in root tissues of *Arabidopsis* (A and C in Figure 2-9). On the contrary, the fluorescent pattern of the HPR2 chimeric protein indicated that it accumulated in the cytosol and the nucleus (E and F in Figure 2-8). These data confirm that HPR1 is transported into leaf peroxisomes because of the presence of the peroxisomal targeting signal, whereas HPR2 is retained in the cytosol because of the lack such a targeting sequence.

Tissue specificity of alternative splicing of HPR in pumpkin

Some proteins produced by alternative splicing show the different expression pattern among tissues. Total extracts from various tissues were investigated whether the two HPR proteins existed (Figure 2-10). HPR was not detected in total extracts from root tissues and cotyledons grown in darkness at both 25°C and 35°C because gene expression of HPR is dependent on light (Figure 2-2). In leaves, flowers and petals, the two HPR proteins exist, such as the case in cotyledons grown in the light. In petals, the amount of polypeptides for HPR was decreased, as accompanying with senescence. This result is in good agreement with that in pumpkin leaves and flowers (De Bellis and Nishimura, 1991; De Bellis et al., 1991; Mano et al., 1996). Interestingly, the polypeptide corresponding to HPR2 strongly dominates in stamen tissues although the total amounts of HPR were very low compared to those in cotyledons and leaves. The amounts of this polypeptide decreased gradually during senescence *in vivo* and were not detectable at 3-day stage from the efflorescence.

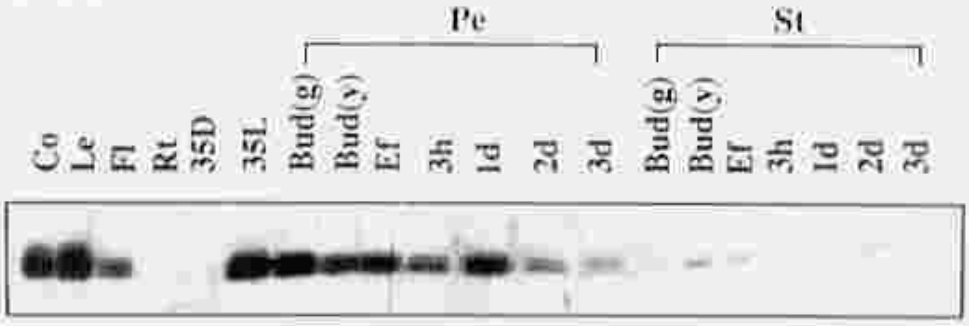


Figure 2-10. Tissue specificity of alternative splicing of HPR in pumpkin

Total protein prepared from various organs was subjected to SDS-PAGE (lane Co to lane 35L, 10μg; other lanes, 20μg), which was followed by immunodetection with antibodies against HPR. Co, 5-day dark- and 4-day light-grown cotyledon; Le, leaf; Fl, the whole flower; Rt, root; 35D, 9-day dark-grown cotyledon at 35℃; 5-day dark- and 4-day light-grown cotyledon at 35℃; Pe, petal; St, stamen; Bud(g) and Bud(y) indicate respectively; petals were green and yellow. Ef, samples at efflorescence; 3h , 1d, 2d and 3d indicate the time of 3 hours, 1 day, 2 day and 3 day, respectively, after efflorescence.

2.5. Discussion

As shown in Figure 1-3, it was anticipated that alternative splicing gave rise to two kinds of HPR mRNA in pumpkin. The Southern blot analysis showed that another HPR gene does not exist in the pumpkin genome (Figure 2-1), demonstrating that the two HPR mRNAs do not originate from different genes. Two mRNAs were certainly expressed (Figure 2-3) because polypeptides corresponding to HPR1 and HPR2 proteins are recognized by an HPR-specific antibody in pumpkin cells (Figure 2-4), indicating that two mRNAs were produced by alternative splicing and end up to different HPR proteins. Interestingly, the two HPR proteins are localized in different parts of cells. HPR1 is in leaf peroxisomes because it is transported on account of the targeting sequence at the carboxy terminus. On the contrary, HPR2 is retained in the cytosol since such a targeting sequence is eliminated by alternative splicing. It is shown that high activity of HPR is detected in the leaf peroxisomes and the cytosol (Figure 2-5). Indirect immunofluorescent microscopy and analyses using transgenic plants, which express each fusion protein containing of ten amino acid residues of the carboxy terminus of either HPR at the end of GFP, revealed different subcellular localizations (Figure 2-6, 2-8 and 2-9). From the comparison with the case of catalase, well defined spots and immunofluorescence over the cell represent the localizations of leaf peroxisomal and cytosolic HPR, respectively. Among higher plants, the small subunit of ADP-glucose pyrophosphorylase (AGPase) in barley, which is a key enzyme of starch biosynthesis, is the only example whose products produced by alternative splicing show the different subcellular localization (Thorbjørnsen et al., 1996). In this case, alternative splicing produces two kinds of the small subunit of AGPase. One is transported into amyloplasts because of the presence of a transit peptide at the amino terminal end and the other remains in the cytosol since alternative splicing eliminates this transit peptide. Some studies have shown that both enzymes are involved in the production of ADP-glucose (Denyer et al., 1996; Thorbjørnsen et al., 1996). In the case of HPR, HPR1 must play a role in photorespiration, but the physiological role of HPR2 remains to be clarified. At present, the metabolic pathway, which involved HPR2, does not seem to be in the cytosol. HPR2

might catalyze the reduction of hydroxypyruvate that escapes from the leaf peroxisomes, since the peroxisomal membrane might not be an absolute barrier to substrates and some overflow of hydroxypyruvate to the cytosol might be expected, especially at times when high light intensity activates the glycolate pathway. In methylotrophs, it has been suggested that HPR plays a dual regulatory step during growth on both C1 and C2 compounds because mutant 20BL, which lacks HPR, is unable to grow on both C1 and C2 compounds (Dunstan et al., 1972). In other higher plants, HPR activities in both leaf peroxisomes and the cytosol have been detected in spinach (Kleczkowski and Randall, 1988), pea leaf protoplasts (Kleczkowski et al., 1988) and barley (Murray et al., 1989), although cDNAs have not been cloned yet. But HPRs in spinach, pea leaf protoplasts and barley seem to be derived from different genes by the following reasoning; (1) the molecular masses of leaf peroxisomal HPR and cytosolic HPR are quite different, (2) each HPR-specific antibody does not cross-react with each polypeptide, (3) peroxisomal HPR uses NADH as the preferred cofactor, whereas cytosolic HPR preferentially uses NADPH instead of NADH. When enzyme activity was measured with NADPH in place of NADH in pumpkin, activities were not detected in the leaf peroxisomal, cytosolic fractions and even in total extracts (data not shown). Murray et al. (1989) discovered a high-CO_2-dependent barley mutant virtually devoid of peroxisomal HPR. In this mutant, the rate of photosynthesis in air was reduced by only about 25% compared with wild-type plants. When this mutant was allowed to assimilate labeled $^{14}CO_2$, the pattern of labeled products clearly demonstrated that substrate was passing through the photorespirate pathway, although there was an abnormal accumulation of label in serine. This result suggests that cytosolic HPR might be responsible for the reduction of hydroxypyruvate. That is, hydroxypyruvate, which escapes from leaf peroxisomes to the cytosol, might be catalyzed to glyoxylate by the reaction of cytosolic HPR and then glyoxylate might be imported to leaf peroxisomes again. Interestingly, HPR2 exists as a major form only in stamen tissues (Figure 2-10), indicating the presence of tissue-specific regulation that contributes to much production of HPR2 mRNA. Further work at mRNA level will be required to characterize the mechanism.

Polypeptides corresponding to HPR1 and HPR2 proteins were detected in the total extracts from pumpkin cotyledons (Figure 2-4). Their amounts were almost the same in the extracts from the dark-grown cotyledons and their accumulations were increased gradually with time. Interestingly, the amounts of both polypeptides, especially HPR2 protein, were induced by light, indicating that gene expression of the two HPR proteins were regulated by light, as is the case with another leaf peroxisomal enzyme, glycolate oxidase (Tsugeki et al., 1993). Light can enhance the accumulation of HPR at mRNA level (Figure 2-2) but a Northern blot analysis can not distinguish HPR1 mRNA from HPR2 mRNA. Based on Figure 2-4, it was anticipated that HPR2 mRNA, but not HPR1 mRNA, was induced dramatically by light. The RT-PCR analysis clearly showed that light had different effects on the induction of the two HPR mRNAs (Figure 2-5). In dark-grown cotyledons at the 5-day stage, the amounts of HPR1 and HPR2 mRNAs were almost the same. However, exposure to continuous light promoted a greater accumulation of HPR2 mRNA than HPR1 mRNA. When the same experiments were performanced several times with different poly(A)$^+$RNA preparations, similar patterns were obtained in all cases. The difference in the accumulation of mRNAs corresponds to that of the two HPR polypeptides, suggesting that light effects the pattern of alternative splicing for HPR. That is, light changes from the production of almost equal amounts of HPR1 and HPR2 mRNAs to mainly production of HPR2 mRNA, although the function of HPR2 in the cytosol and the mechanism of much production of the HPR2 mRNA are unknown. Bertoni and Becker (1996) demonstrated that a phytochrome-component is involved in the induction of total HPR mRNA in dark-adapted cucumber cotyledons by red-light induction and partial far-red-light reversity. Same components might take part in the regulation of alternative splicing for pumpkin HPR. This is the first proof that alternative splicing, which is regulated by light, produces the isoforms whose subcellular localizations are different. Our results indicate the novel mechanism of the microbody transition that is regulated by alternative splicing.

Chapter 3

Molecular cloning and characterization of hydroxypyruvate reductase in *Arabidopsis thaliana*

3.1. Summary

Five *Arabidopsis* EST cDNA clones of hydroxypyruvate reductase (HPR) were sequenced. Deduced amino acid sequences revealed that HPR in *Arabidopsis* contained the carboxy terminal targeting signal to microbodies. Nucleotide sequence analysis showed that the cDNA with the longest insert contained an open reading frame of 1,158 bp which encoded a polypeptide with 386 amino acids with a calculated molecular mass of 42,251 Da. A Southern blot analysis suggested that the *Arabidopsis* HPR gene, like that of the pumpkin HPR gene, exists as a single copy. Judging from the structure of genomic DNA, *Arabidopsis* HPR gene might not undergo alternative splicing because sites for alternative splicing did not exit, such as did pumpkin. It is sure that one polypeptide with a molecular mass of 42 kDa is detected in *Arabidopsis* leaves. In addition, immunoelectron microscopy revealed that *Arabidopsis* HPR protein was exclusively localized in leaf peroxisomes in green leaves. These results indicate that HPR is expressed in a form with a carboxy terminal targeting signal to microbodies and is localized in microbodies in *Arabidopsis*, suggesting that the differences in the gene structure and regulation of gene expression of HPR are probably due to species-specific differences in plants.

3.2. Introduction

As demonstrated in Chapter 2, there are two kinds of HPR proteins produced by alternative splicing in pumpkin. To date, cDNAs for HPR have been cloned from pumpkin (Hayashi et al., 1996) and cucumber (Greenler et al., 1989) in higher plants. In the case of cucumber, the cDNA encodes HPR without the carboxy terminal targeting signal to microbodies. However, the analysis of HPR gene of cucumber suggested the involvement of alternative splicing (Hayashi et al., 1996), indicating that cucumber HPR with the microbody targeting signal exists, although the cDNA has not been cloned. Therefore, it is of interest to analyze the structure and expression of HPR in another plant species. For this purpose, cDNA cloning and characterization of *Arabidopsis* HPR was

performed in order to investigate whether *Arabidopsis* HPR has the targeting signal to microbodies, by screening the Arabidopsis Biological Resource Center (ABRC) at Ohio State University . Five EST clones homologous to pumpkin HPR have been already registered and were available. In this Chapter, the analysis of characterization of *Arabidopsis* HPR is described. In addition, the presence of two HPR proteins were examined in various plants.

3.3. Materials and methods

Plant materials

Seeds of *Arabidopsis thaliana* (L.) Heynh. (ecotype Columbia, C24 and Lansberg *erecta*) were grown at 22°C in darkness. Pumpkin (*Cucurbita* sp. cv. Kurokawa Amakuri Nankin), cucumber (Kagafushinari and Chikanarisuiyoh) and watermelon seeds were soaked overnight and germinated in moist rock fiber (66R; Nitto Bouseki, Chiba, Japan) at 25°C under continuous light. Leaves of spinach, carrot , rice and *Chrysanthemum coronarium* were kindly provided by Ms. M. Ohtake.

Sequencing of *Arabidopsis* HPR EST clones

Five *Arabidopsis* HPR EST clones, whose stock numbers were 60A7T7, 135P15T7, 113J10T7, 183L19T7 and 133M20T7, were obtained from ABRC. These were derived from a λZip-Lox plasmid (GIBCO BRL, MD, USA). The nucleotide sequences encoding around the carboxy terminal region of each clone and the full-length sequence of the longest cDNA insert, designated 113J10T7, were determined with an automatic DNA sequencer (model 373; Perkin Elmer/Applied Biosystems) according to the manufacturer's instructions. The nucleotide and deduced amino acid sequences were analyzed with DNA analytical software (GeneWorks; IntelliGenetics, Mountain View, CA)

Cloning of genomic DNA for *Arabidopsis* HPR

An intron of the gene for *Arabidopsis* HPR was amplified by polymerase chain reaction (PCR) using total genomic DNA (5 ng) as a template. The genomic DNA was extracted from *Arabidopsis* leaves by the cetyltrimethylammonium bromide precipitation method (Murray and Thompson, 1980). The reaction mixture contained 1 unit of Ampli Taq DNA polymerase (Perkin Elmer Japan, Chiba, Japan), an upstream primer (ATHPR1; 5'-GCATCGTCAACTCAAAGGCCTTA-3'), a downstream primer (ATHPR2; 5'-TACATAAACACAACTTGACACC-3') and an appropriate buffer in a total volume of 50 µl. Each cycle was at 94 °C for 45 sec, 55 °C for 45 sec and 72 °C for 45 sec. The DNA fragment was subcloned into a T-vector prepared using pBluescript KS^+ as described in a previous report (Marchuk et al., 1990).

Southern blot analysis

Total DNA (1 µg) was digested with *Bgl*II and *Xba*I. The products were fractionated on a 0.8% agarose gel, transferred to a Zeta-Probe blotting membrane (BIO-RAD, CA, USA) by the capillary method and fixed by exposure to UV-light (Funa-UV-Linker, model FS-800; Funakoshi, Tokyo, Japan). The fragment corresponding to *Arabidopsis* HPR EST clone (113J10T7) was labeled with [^{32}P]-dCTP (Amersham Japan) using a *Bca*Best labeling kit (Takara Shuzo, Kyoto, Japan). The membrane was hybridized in 50% formamide, 0.12 M sodium phosphate (pH 7.2), 0.25 M sodium chloride, 7% SDS and 1mM EDTA (pH 8.0) with 1.0×10^6 cpm·ml^{-1} of radiolabeled DNA probe for 18 h at 42°C. The membrane was washed at 42°C in 2 × SSC plus 0.1% SDS for 15 min, in 0.2 × SSC plus 0.1% SDS for 15 min, in 0.1 × SSC plus 0.1% SDS for 15 min. The membrane was used to expose X-ray film.

Immunoblot analysis

The leaves of *Arabidopsis* plants at the 21-old-day stage and various species, and cotyledons of pumpkin, cucumber and watermelon were homogenized with the extraction buffer (100mM Tris-HCl, pH 6.8, 1mM EDTA, pH 8.0, 0.1% Triton X-100 and 1mM PMSF) and then the homogenates were centrifuged at 15,000 × *g* for 25 min. The protein

content of each extract was estimated by using a protein assay kit (Nippon Bio-Rad Laboratories, Tokyo, Japan) with bovine gamma albumin as a standard protein. Total protein was separated by SDS-PAGE on a 7.5% polyacrylamide gel as described by Laemmli (1970) and transferred to a nylon membrane (Schleicher & Schuell, Dassel, Germany) in a semidry electroblotting system. Immunologic reactions were detected by monitoring the activity of horseradish peroxidase antibodies against rabbit IgG (ECL system; Amersham, Japan).

Immunoelectron microscopy

Arabidopsis leaves (ecotype Columbia) at the 21-day-old stage were fixed, dehydrated and embedded in LR white resin (London Resin, UK) as described previously (Nishimura et al., 1993). Ultra-thin sections were cut on a Reichert ultramicrotome (Leica, Heidelberg, Germany) with a diamond knife and mounted on uncoated nickel grids. The protein A-gold labeling procedure was essentially the same as that described by Nishimura et al. (1993). Ultra-thin sections were incubated at room temperature for 1 h with a solution of antiserum against HPR diluted 1:2000 and then with a 50-fold diluted suspension of protein A-gold (Amersham Japan) at room temperature for 30 min. The sections were examined with a transmission electron microscope (1200EX; JOEL, Tokyo, Japan) at 80 kV.

3.4. Results

Full-length sequence of *Arabidopsis* HPR cDNA

Of five EST clones, the complete nucleotide sequence of 113J10T7, which contained the longest insert, was determined. As shown in Figure 3-1, this cDNA consists of 1,428 bp and contains a 1,158-bp open reading frame that encodes a polypeptide with 386 amino acids with a total molecular mass of 42,251 Da. The deduced sequence beginning with the first ATG at nucleotide 81 showed high similarities to HPRs from other sources such as pumpkin (Hayashi et al., 1996), cucumber (Greenler et al.,

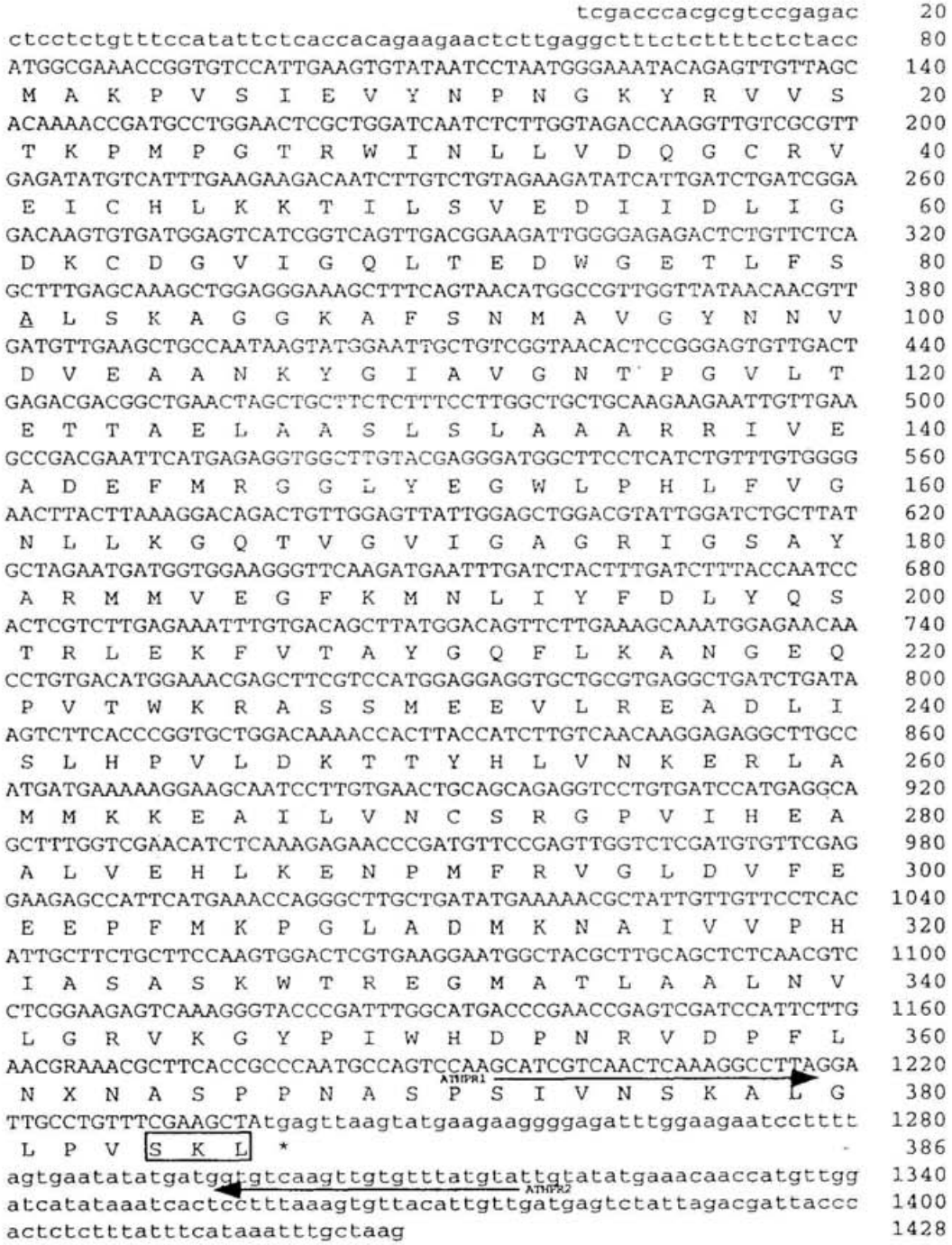


Figure 3-1. Nucleotide and deduced amino acid sequences of *Arabidopsis* HPR cDNA

The complete nucleotide sequence of 113J10T7 was determined. The nucleotide sequence of the putative coding region is shown in uppercase letters and the 5'- and 3'-noncoding regions are shown in lowercase letters. The deduced amino acid sequence is presented in the single-letter code under the nucleotide sequence, starting at the first in-frame methionine residue and ending at the first stop codon, indicated by an asterisk. The three carboxy-terminal amino acids that are known as a microbody-targeting signal are boxed. Arrows indicate primers, ATHPR1 and ATHPR2, for PCR, respectively.

1989) and methylotrophs (Chistoserdova and Lidstrom, 1994; Yoshida et al., 1994). The identities with amino acid sequences of HPR for other plants are 88% for pumpkin HPR1, 87% for pumpkin HPR2 and 87% for cucumber (data not shown). It is noteworthy of the presence of tripeptide, Ser-Lys-Leu, at the carboxy terminus.

Nucleotide sequences around the carboxy terminus of *Arabidopsis* EST clones

The nucleotide sequences encoding the region around the carboxy terminus of other clones were determined in order to investigate whether these sequences contained the targeting signal to microbodies. As shown in Figure 3-2, the nucleotide sequences of all cDNAs were identical, although the lengths and positions of the poly(A) tail were different. All deduced amino acid sequences contained the Ser-Lys-Leu sequence at their carboxy termini and none of the HPRs were found to lack the targeting signal, as occurs in pumpkin HPR2 cDNA.

Genomic sequence and putative amino acid sequence of *Arabidopsis* HPR

To characterize the structure of *Arabidopsis* HPR gene, partial *Arabidopsis* genomic DNA was amplified by PCR. A unique fragment of about 300 bp in length was amplified, subcloned into pBS vector and then subjected to sequence analysis. The result showed a 188-bp intron located at the same position as it is in the HPR genes of pumpkin and cucumber (Figure 3-3). An exon-intron junction followed the GT-AG rule and showed good agreement with the donor and acceptor consensus sequence. There were not two pairs of GT-AG doublets within this intron. The nucleotide sequences of six independently isolated products of PCR amplified from the genomic DNA were sequenced but all these DNAs had the same nucleotide sequence as shown in Figure 3-3. These data suggest that the gene for *Arabidopsis* HPR contains only one GT-AG doublet and that alternative splicing might not occur.

```
                  L  G  L  P  V  S  K  L
60A7T7          CCTTAGGATTGCCTGTTTCGAAGCTAtgagttaagtatgaagaaggggag
135P15T7        CCTTAGGATTGCCTGTTTCGAAGCTAtgagttaagtatgaagaaggggag
113J10T7        CCTXAGGATTGCCXGTTTCGAAGCTAtgagttaagtatgaagaaggggag
183L19T7        CCTXAGGATTGCCXGTTTCGAAGCTAtgagttaagtatgaagaaggggag
113M20T7        TTTCAGGATTGCCTGTTTCGAAGCTAtgagttaagtatgaagaaggggag
                  * ********* ************************************

60A7T7          atttggaagaatccttttagtgaatatatgatggtgtcaagttgtgttta
135P15T7        atttggaagaatccttttagtgaatatatgatggtgtcaagttgtgttta
113J10T7        atttggaagaatccttttagtgaatatatgatggtgtcaagttgtgttta
183L19T7        atttggaagaatccttttagtgaatatatgatggtgtcaagttgtgttta
113M20T7        atttggaagaatccttttagtgaatatatgatggtgtcaagttgtgttta
                ****************************************************

60A7T7          tgtattgtatatgaaacaaccatgttggatcatataaatcactcctttaa
135P15T7        tgtattgtatatgaaacaaccatgttggatcatataaatcactcctttaa
113J10T7        tgtattgtatatgaaacaaccatgttggatcatataaatcactcctttaa
183L19T7        tgtattgtatatgaaacaaccatgttggatcatataaatcactcctttaa
113M20T7        tgtattgtatatgaaacaaccatgttggatcatataaatcactcctttaa
                ****************************************************

60A7T7          agtgttacattgttgatgagtctattagacgattacccactctctttatt
135P15T7        agtgttacattaaaaaaaaaaaaaa
113J10T7        agtgttacattgttgatgagtctattagacgattacccactctctttatt
183L19T7        agtgttacattgttgatgagtctattagacgattacccactctctttatt
113M20T7        agtgttacattgttgatgagtctattagacgattacccactctctttatt
                ***********

60A7T7          tcataaatttgctaagaatacaatttctaagtcaaaaaaaaaaaaaaaaa
113J10T7        tcataaatttgctaagaatacaaaaaaaaaaaaaaaaaaaaaa
183L19T7        tcataaatttgctaagaatacaaaaaaaaaaaaaaaaaaaaaa
113M20T7        tcataaatttgctaagaatacaatttctaagtcatcaagaaaaaaaaaaa

60A7T7          aaa
113M20T7        aaaaaaaaaaaa
```

Figure 3-2. Alignment of nucleotide sequences encoding the carboxy-terminal amino acids of *Arabidopsis* EST clones

Each nucleotide sequence for the coding region is shown in uppercase letters and the 3'-noncoding region is shown in lowercase letters. The deduced amino acid sequence around the carboxy terminus is presented in the single-letter code over the nucleotide sequence. Identical nucleotides are indicated by an asterisk under the sequence. The stock number of each clone is shown on the left.

(A) GCATCGTCAACTCAAAGGCCTTAGgtactacacaacacttacactcatgc

I V N S K A L G

ctcagattgtttcaaacgctaatttatgcttgcatttcctgttaacaaaa
tatttaaacataaacagaaacaaaagaacattgactaatccacttaaaca
aatttgttcccattctctctattaaaattactctcatatatatattcttt
gggtttttcagGATTGCCTGTTTCGAAGCTAtgagttaagtatgaagaa

L P V S K L *

ggggagatttggaacaatccttttagtgaatatatgatggtgtcaagttg
tgtttatgt

(B)

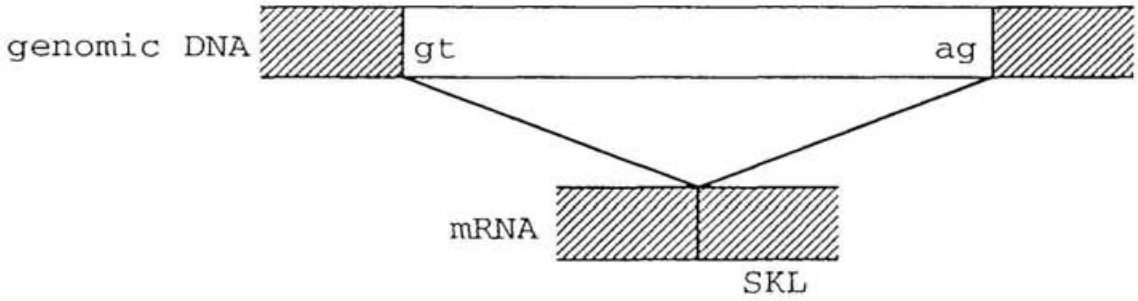


Figure 3-3. Structural relationships around the carboxy terminal end of HPR from *Arabidopsis*

(A) The nucleotide sequence of the intron in this region of *Arabidopsis* HPR gene is shown. The nucleotide sequence of the exon is shown in uppercase letters and the intron and 3'-noncoding region are shown in lowercase letters. The deduced amino acid sequence is presented in the single-letter code under the nucleotide sequence. Asterisk indicates the stop codon and boxes represent the consensus sequences found in the beginning (GT) and the end (AG) of an intron. (B) Schematic representation between HPR mRNA and the gene in *Arabidopsis*. The hatched boxes correspond to exons and the open boxes correspond to the intron. The consensus sequences corresponding to the splice donor and acceptor sites and the carboxy-terminal amino acids are shown.

Arabidopsis HPR is encoded by a single copy gene

A Southern blot analysis was carried out to examine whether another *Arabidopsis* HPR gene exists. Genomic DNA from *Arabidopsis* leaves was isolated, digested with *Bgl*II and *Xba*I, none of which cuts *Arabidopsis* cDNA (113J10T7), and subjected to a Southern blot hybridization using *Arabidopsis* HPR cDNA as a probe. As shown in Figure 3-4, HPR cDNA hybridized with a single fragment. In addition, only one kind of genomic sequence was obtained when the genomic DNA was amplified (Figure 3-3). These findings indicate that HPR in *Arabidopsis* exists as a single-copy gene, as it does in pumpkin and cucumber.

Arabidopsis HPR is exclusively localized in leaf peroxisomes

The localization of HPR protein was investigated using immunoelectron microscopy. Gold particles for HPR were exclusively detected in leaf peroxisomes but not in other organelles such as mitochondria, chloroplasts and vacuoles (Figure 3-5). This electron micrograph clearly shows that HPR is transported into leaf peroxisomes in *Arabidopsis*. This result indicates that *Arabidopsis* HPR is localized in only leaf peroxisomes as a form with a targeting signal to microbodies.

Antibodies against HPR cross-react with a 42 kDa polypeptide

Immunoblotting was carried out using antibodies against spinach HPR. Total proteins prepared from *Arabidopsis* and pumpkin, as a reference, were subjected to SDS-PAGE. A unique polypeptide with a molecular mass of 42 kDa was recognized by the antibodies against spinach HPR in the total *Arabidopsis* extract (lane 1 and 2 in Figure 3-6). This is in good agreement with the molecular mass calculated from the deduced amino acids. In the case of total protein from pumpkin, two polypeptides were detected (lane 3 in Figure 3-6). This unique band in *Arabidopsis* did not split into two bands under the same electrophoretic condition, even though the volume of total protein was reduced (Figure 3-6, lane 1). This data indicated that HPR in *Arabidopsis* is only expressed as a 42 kDa polypeptide.

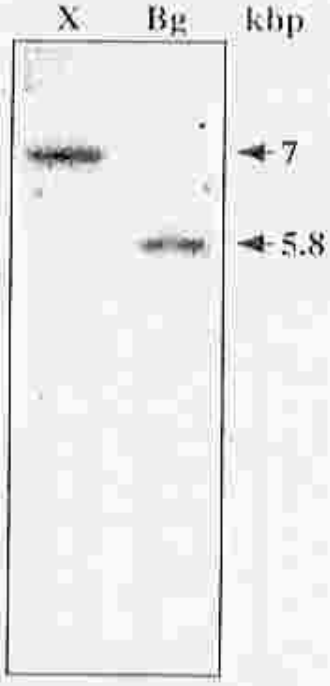


Figure 3-4. Southern blot analysis of *Arabidopsis* genomic DNA

Genomic DNA (1 μg each) was digested with the restriction endonucleases indicated, separated on a 0.8% agarose gel, transferred to a Zeta-Probe blotting membrane (BIO-RAD) and probed with a [^{32}P]-labeled *Arabidopsis* HPR cDNA fragment. The sizes of DNA fragments are shown in kbp on the right. X, *Xba*I; Bg, *Bgl*II.

Figure 3-5. Immunocytochemical localization of HPR in *Arabidopsis*.

Immunogold labeling of ultra-thin sections of *Arabidopsis* leaves was carried out using antibodies against HPR. Mb, microbody; Mt, mitochondrion; Ch, chloroplast; V, vacuole. Bar indicates 1 μm.

Figure 3-6. Detection of HPR polypeptides in *Arabidopsis* and pumpkin.

Total extracts prepared from *Arabidopsis* leaves and pumpkin cotyledons were subjected to SDS-PAGE, which were followed by immunodetection with antibodies against HPR. Lane 1 and 2, 5 µg and 10 µg of total extracts of *Arabidopsis*, respectively; lane 3, 10 µg of total extract of pumpkin cotyledons. Molecular markers are indicated in kDa on the left. Arrows on the right represent the HPR1 and HPR2 polypeptides of pumpkin.

Immunochemical characterization of HPR from various plant tissues

Pumpkin has the two HPR proteins and *Arabidopsis* (ecotype Columbia) has only one HPR that is localized in leaf peroxisomes. Then how about another plants? To address this question, immunodetection of HPR was carried out using extracts from various plants (Figure 3-7). As expected by Hayashi et al. (1996), two electrophoretically similar polypeptides were detected in the total extracts of cucumber, implying the presence of two HPR proteins, although their localizations have not been determined yet. However, only one polypeptide was detected in rice and another *Arabidopsis* (ecotype C24 and Lansberg *erecta*) in addition to watermelon, another oilseed plant. Antibodies against spinach HPR did not cross to the polypeptides in the extracts from carrot and Chrysanthemum coronarium. In the extracts from spinach, one major band, which is smaller than pumpkin HPRs, and a few faint bands were detected. This result is in good agreement with the results by Kleczkowski and Randall (1988).

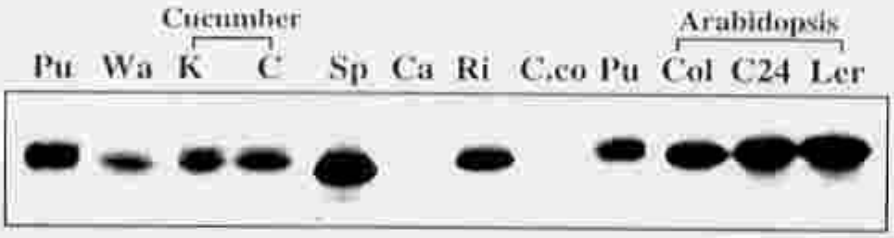


Figure 3-7. Immunodetection of HPR proteins from leaves of different species.

Ten μg of total extracts prepared from leaves of different species were subjected to SDS-PAGE, which were followed by immunodetection with antibodies against HPR. Pu; pumpkin, Wa; watermelon, K; cucumber (Kagafushinari), C; cucumber (Chikanarisuiyoh), Sp; spinach, Ca; carrot, Ri; rice, C.co; *Chrysanthemum coronarium*, Col; *Arabidopsis* (ecotype Columbia), C24; *Arabidopsis* (ecotype C24), Ler; *Arabidopsis* (ecotype Lansberg *erecta*).

3.5. Discussion

An EST bank at ABRC at Ohio State University was screened for HPR cDNA in *Arabidopsis* using the amino acid sequence of pumpkin HPR1 protein. Five cDNA clones showed high similarities to pumpkin HPRs. At the result of the determination of nucleotide sequences encoding around the carboxy termini (Figure 3-2), all clones have the Ser-Lys-Leu peptide at the carboxy termini, demonstrating that they encode the HPR1-type proteins and that *Arabidopsis* HPR is localized in leaf peroxisomes. The Southern blot analysis and the determination of nucleotide sequence of the intron were carried out to investigate the possibility of the presence of another gene. These data revealed that *Arabidopsis* HPR was a single-copy gene (Figure 3-4) and that only one pair of the GT-AG doublet existed in the intron (Figure 3-3), suggesting that alternative splicing of HPR pre-mRNA might not undergo in *Arabidopsis*. Givan and Kleczkowski (1992) detected the activities and polypeptides of HPR in leaf peroxisomes and the cytosol of barely leaves and Kleczkowski reported the cytosolic and leaf peroxisomal activities for HPR in spinach, pea and barley (Kleczkowski and Randall, 1988; Kleczkowski et al., 1988; Kleczkowski and Edwards, 1989), namely, besides pumpkin, another plants appear to have the leaf peroxisomal and cytosolic HPR proteins, although they seem to be derived from different genes as described in Discussion of Chapter 2. Then how about other plants and another ecotypes of *Arabidopsis*? To clarify this question, immunochemical analysis was performed using the extracts from various plants (Figure 3-7). As Hayashi et al. (1996) expected, two polypeptides were recognized in the extract from cucumber, since the HPR gene of cucumber has two pairs of the GT-AG doublets as does pumpkin HPR gene, suggesting that alternative splicing results in the two HPR proteins. This data is in good agreement with our speculation, although the subcellular localization remains to be determined. One polypeptide was detected in the extract from watermelon that is one of oilseed plants such as pumpkin and cucumber, suggesting that this plant has one HPR or another HPR that antibodies against spinach HPR is unable to crossreact. Moreover, one immunopositive polypeptide was recognized in the extracts from rice and another ecotypes of *Arabidopsis*. This result indicates that all

tested *Arabidopsis* have the leaf peroxisomal HPR and that it does not depend on the difference between monocots and dicots. As stated above, the cytosolic and leaf peroxisomal HPR seem to originate from different gene so that each antibody might not crossreact with each other. Figure 3-7 probably shows the detection of leaf peroxisomal HPR. Our data shows the possibility that the differences in gene structure and regulation of gene expression of HPR are probably due to be species-specific manner in plants. It is worthy to note the different amounts between polypeptides in cucumber, and the amount of lower polypeptide corresponding to HPR2 is more than that of upper one corresponding to HPR1. This result is in accord with the case of pumpkin, although the physiological function of HPR2 is unknown.

Chapter 4

Molecular cloning and characterization of an alternatively spliced chloroplastic ascorbate peroxidase in pumpkin, stromal ascorbate peroxidase

4.1. Summary

A cDNA encoding stromal ascorbate peroxidase (sAPX) was isolated using poly(A)$^+$RNA of pumpkin cotyledons by RT-PCR. The cDNA encodes a polypeptide with 372 amino acids and shares complete sequence identity with pumpkin thylakoid-bound ascorbate peroxidase (tAPX), except for the deletion of a putative membrane spanning region located in the carboxy domain of tAPX. From a Southern blot hybridization, pumpkin chloroplastic APX exists as a single copy. Moreover, analysis of genomic structure showed the presence of one donor site and two acceptor sites in the region encoding around the carboxy domain, indicating that mRNAs for sAPX and tAPX are produced by alternative splicing. An immunoblot analysis demonstrated that the accumulation of sAPX and tAPX proteins was differently regulated during germination and subsequent greening of pumpkin cotyledons, suggesting that alternative splicing of pumpkin chloroplastic APXs might be regulated by light, as is pumpkin HPR.

4.2. Introduction

As mentioned in Chapter 1.4, plant cells have four kinds of APX with different subcellular and suborganellar localizations. Of these, information about chloroplastic APXs is restricted to only very few example, although it has been reported that chloroplastic APXs function in the scavenging of photoproduced hydrogen peroxide. To date, chloroplastic APXs has been purified, characterized, and their activities were estimated (Chen and Asada, 1989; Chen et al., 1992; Miyake et al., 1993; Miyake and Asada, 1992). With regard to cDNA cloning, in our laboratory, cDNA for tAPX in pumpkin has been already cloned by Yamaguchi et al. (1996). From its nucleotide sequence, the crossreactivity of polypeptides corresponding to tAPX and sAPX proteins and the partial amino acid sequence of the N-terminal of sAPX polypeptide, it was anticipated that alternative splicing might regulate the production of sAPX and tAPX mRNAs. Moreover, it was suggested that light might control the alternative splicing because the development of chloroplasts depends on light. However, information about

their speculations have not been obtained. For the purpose of investigating whether sAPX and tAPX are produced by alternative splicing, cDNA for sAPX in pumpkin was cloned from greening pumpkin cotyledons and characterized.

4.3. Materials and methods

Plant materials

Pumpkin (*Cucurbita* sp. cv. Kurokawa Amakuri Nankin) seeds were soaked overnight and germinated in moist rock fiber (66R; Nitto Bouseki, Chiba, Japan) at 25°C in darkness. Some of these seedlings were transferred to continuous illumination at the 5-day stage.

Sequencing of cDNA for pumpkin sAPX

First-strand cDNA was generated using a Ready-To-Go T-primed first-strand kit (Pharmacia Biotech, Tokyo, Japan) from poly(A)$^+$ RNA template prepared from 5-day dark- and 4-day light-grown cotyledons using Oligotex-dT 30 <Super> (Roche Japan, Tokyo, Japan). PCR was used to isolate full-length cDNA for pumpkin sAPX. The reaction mixture contained 1 unit of Ampli Taq DNA polymerase (Perkin Elmer Japan, Chiba, Japan), an upstream primer (ASA-S1; 5'-ATCGCGTTCATTGCCAGTTG-3'), a downstream primer (SAPX-II; 5'-GACACGGTCACAACAAAACATG-3') that was synthesized corresponding to the genomic sequence obtained previously (Figure 4-1) and an appropriate buffer in a total volume of 50 µl. Each cycle was at 94 °C for 45 sec, 55 °C for 45 sec and 72 °C for 45 sec. The DNA fragment was subcloned into a T-vector prepared using pBluescript KS$^+$ as described previously (Marchuk et al., 1990). The nucleotide sequence was determined with an automatic DNA sequencer (model 377; Perkin Elmer/Applied Biosystems) according to the manufacturer's instructions. The nucleotide and the deduced amino acid sequences were analyzed with DNA analytical software (GeneWorks; IntelliGenetics, Mountain View, CA)

Cloning of genomic DNA for pumpkin sAPX

An intron of the gene for pumpkin sAPX (tAPX) was amplified by PCR using total genomic DNA (5 ng) as a template. The genomic DNA was extracted from pumpkin leaves by the cetyltrimethylammonium bromide precipitation method (Murray and Thompson, 1980). The reaction mixture contained 1 unit of Ampli Taq DNA polymerase (Perkin Elmer Japan, Chiba, Japan), an upstream primer (pumpchl A; 5'-GGTATTGTGATTGATGATGC-3'), a downstream primer (3'-non; 5'-GGATCGTGAAATCAGAAGACA-3') and an appropriate buffer in a total volume of 50 µl. Each cycle was at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min. Cloning and sequencing of the DNA were carried out as described above.

Southern blot analysis

Total DNA (10 µg) was digested with *Mlu*I, *Sal*I, *Xho*I and *Bam*HI. The products were fractionated on a 0.8% agarose gel, transferred to a Zeta-Probe blotting membrane (BIO-RAD, CA, USA) by the capillary method and fixed by exposure to UV-light (Funa-UV-Linker, model FS-800; Funakoshi, Tokyo, Japan). The DNA fragment corresponding to pumpkin sAPX was labeled with [^{32}P]-dCTP (Amersham Japan) using a Megaprime DNA labeling system (Amersham Japan). The membrane was hybridized in 50% formamide, 0.12 M sodium phosphate (pH 7.2), 0.25 M sodium chloride, 7% SDS and 1mM EDTA (pH 8.0) with 1.0×10^6 cpm·ml^{-1} of radiolabeled DNA probe for 18 h at 42°C. The membrane was washed at 42°C in 2 × SSC plus 0.1% SDS for 15 min, in 0.2 × SSC plus 0.1% SDS for 15 min, in 0.1 × SSC plus 0.1% SDS for 15 min. The membrane was used to expose to the imaging plate of a Bio-Imaging analyzer (FUJIX BAS2000; FUJI Photo Film, Tokyo) with an exposure time of 18 h.

RT-PCR analysis

First-strand cDNA was synthesized using poly(A)$^+$ RNA prepared from 5-day dark- and 2-day light-grown cotyledons. PCR was performed as described previously except for EX Taq DNA polymerase (Takara Shuzo, Kyoto, Japan) and specific primers;

the 5'-common sense primer of sAPX and tAPX (ASA3; 5'-CCTGGACTGTACAATGG-3') and the 3'-specific antisense primers for tAPX (3'-non; shown previously) and sAPX (SAPX-I; 5'-AGACGCGGACATCACAGATGTC-3'). The condition was 25 cycles of 45s at 94°C, 45s at 52°C and 45s at 72°C.

Immunoblot analysis

Pumpkin cotyledons grown under various stages and different tissues were homogenized with SDS-loading buffer (50mM Tris-HCl, pH 8.0, 1mM EDTA, pH 8.0, 0.2mM NaCl and 10% SDS) and then the homogenates were centrifuged at 12,000 × rpm for 10 min. Supernatants were subjected to SDS-PAGE on a 10% polyacrylamide gel as described by Laemmli (1970) and transferred to a nylon membrane (Schleicher & Schuell, Dassel, Germany) in a semidry electroblotting system. Antibodies against pumpkin mbAPX prepared as described previously (Yamaguchi et al., 1995) were used and the polypeptides on the membrane were visualized with 1: 5,000 dilution of horseradish peroxidase antibodies against rabbit IgG (ECL system; Amersham Japan).

4.4. Results

Nucleotide sequence analysis

The RT-PCR was performed to amplify the coding region of pumpkin sAPX using primers of ASA-S1 and SAPX-II (Figure 4-1). The 1.2-kb product was cloned into pBS vector and sequenced. The cDNA consists of 1,208 bp and contains a 1,116-bp open reading frame that encodes a polypeptide with 372 amino acids with a total molecular mass of 40,661 Da. The amino terminal amino acids segment shows typical characteristics of a chloroplast targeting signal, i.e. high content of hydroxylated amino acids and low proportion of acidic ones (Karlin-Neumann and Tobin, 1986). In the case of cleavage of the transit peptide on the C-terminal side of Cys-77, the calculated total molecular mass is 32,594 Da. This is in good agreement with the molecular mass of polypeptides recognized by antibodies against pumpkin mbAPX (Yamaguchi et al.,

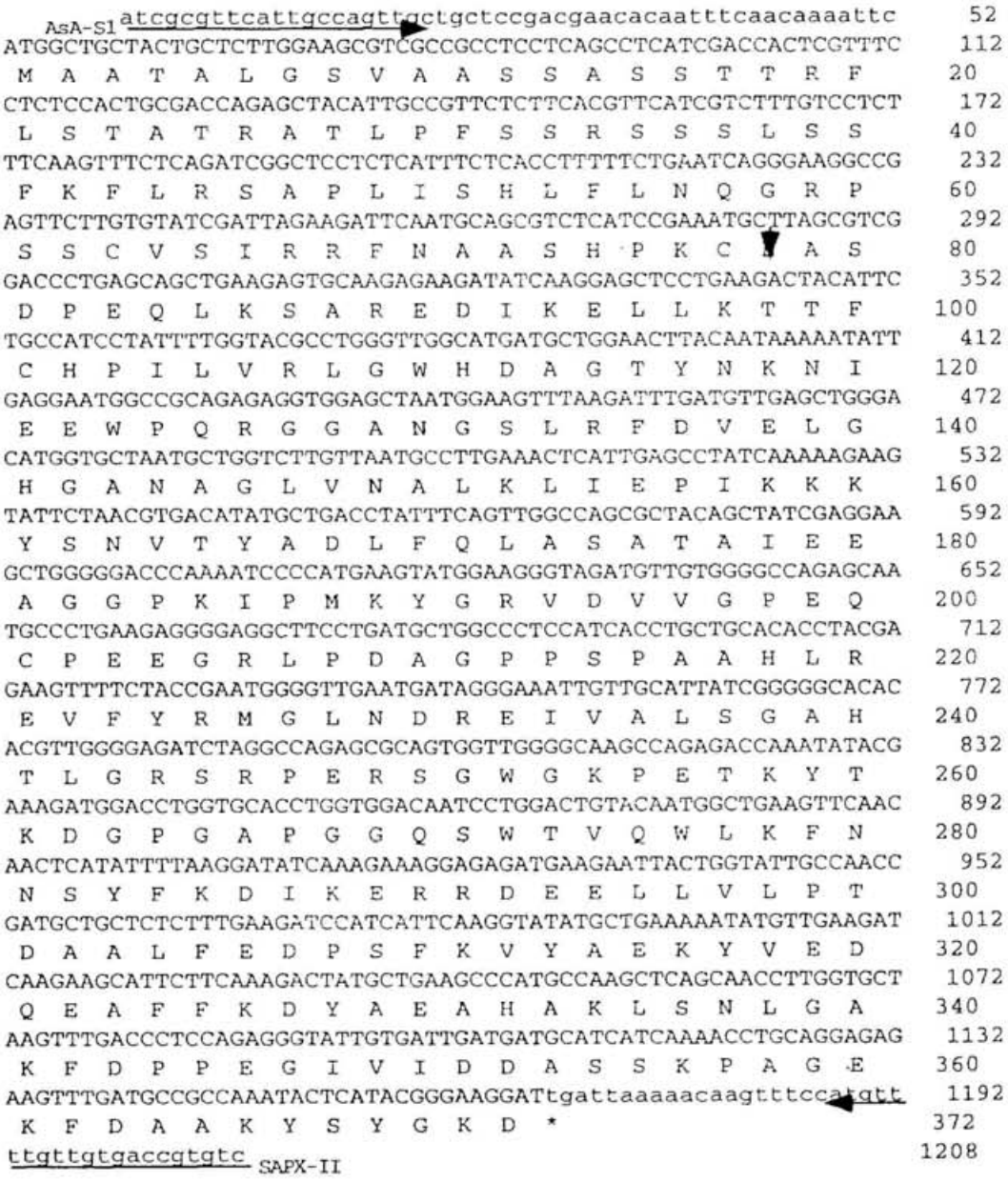


Figure 4-1. Nucleotide and deduced amino acid sequence of pumpkin sAPX cDNA

The nucleotide sequence of the putative coding region is shown in uppercase letters and the 5'- and 3'-noncoding regions are shown in lowercase letters. The deduced amino acid sequence is presented in the single-letter code under the nucleotide sequence, starting at the first in-frame methionine residue and ending at the first stop codon, indicated by an asterisk. Arrowhead indicates the putative site of cleavage of the transit peptide, as estimated from the N-terminal sequence of tea sAPX and spinach tAPX. Primers for PCR (ASA-S1 and SAPX-II) are underlined with an arrowhead indicating the polarity.

1995). The deduced amino acid sequence from pumpkin sAPX cDNA was 76% identical with spinach sAPX. The sequence of cDNA showed complete identity with that of pumpkin tAPX cDNA (Yamaguchi et al., 1996) except for the carboxy terminal region (Figure 4-2, A and B), which contains a putative thylakoid-spanning domain (Figure 4-3, C).

Genomic structure of pumpkin chloroplastic APX

The PCR-amplified genomic sequence around the carboxy terminal region was determined to investigate whether sAPX and tAPX in pumpkin were also produced by alternative splicing. A unique fragment of about 550 bp in length was amplified, subcloned into pBS vector and sequenced. The sequence showed the presence of a 291-bp intron that contains one donor site and two acceptor sites (Figure 4-3, A), suggesting that the synthesis of sAPX mRNA or tAPX mRNA depends on which acceptor site is used for the splicing (Figure 4-3, B). The nucleotide sequences of independently isolated products of PCR amplified from the genomic DNA were the same as shown in Figure 4-3 (A).

Chloroplastic APXs are encoded by a single gene

To investigate whether another sAPX gene exists, the genomic DNA from pumpkin leaves was isolated, digested with *Mlu*I, *Sal*I and *Xba*I (none of which cuts pumpkin sAPX cDNA) and with *Bam*HI (which cuts the cDNA at one site) and then subjected to a Southern blot hybridization using pumpkin sAPX cDNA as a probe. As shown in Figure 4-4, the sAPX probe hybridized to single bands when digested with *Mlu*I, *Sal*I and *Xba*I, while it gave two bands when digested with *Bam*HI. In addition, only one kind of genomic sequence was obtained when the genomic DNA was amplified (Figure 4-3, A). These findings indicate that sAPX in pumpkin exists as a single-copy gene and alternative splicing produces two kinds of mRNAs for sAPX and tAPX.

(A)

```
sAPX    FDAAKYSYGKD-----------------------------------------------
tAPX    FDAAKYSYGKRELSDSMKQKIRAEYESFGGSPDKPLPTNYFLNIILVIAVLAILTSLLGN
```

(B)

```
sAPX    CCTGCAGGAGAGAAGTTTGATGCCGCCAAATACTCATACGGGAAGGATTGATTAA-AAAC
tAPX    CCTGCAGGAGAGAAGTTTGATGCCGCCAAATACTCATACGGGAAGAGAGAGCTATCAGAT

sAPX    AAGTTTCCATGTTTTGTTGTGACCGTGTC-----------------------------
tAPX    TCAATGAAGCAGAAGATTCGGGCGGAATACGAAAGCTTTGGTGGAAGTCCAGATAAGCCT

sAPX    ------------------------------------------------------------
tAPX    TTACCAACAAACTACTTCCTTAATATCATACTTGTGATTGCTGTTTTGGCAATTTTGACA

sAPX    ------------------------------------------------------------
tAPX    TCTCTTCTAGGAAACTGAGGTTTTGAATCTACCTTTATTTCTCAACACAATGTCTTCTGA

sAPX    ------------------------------------
tAPX    TTTCACGATCCATTTCTAATGCTTACCTACTTGAAATT
```

(C)

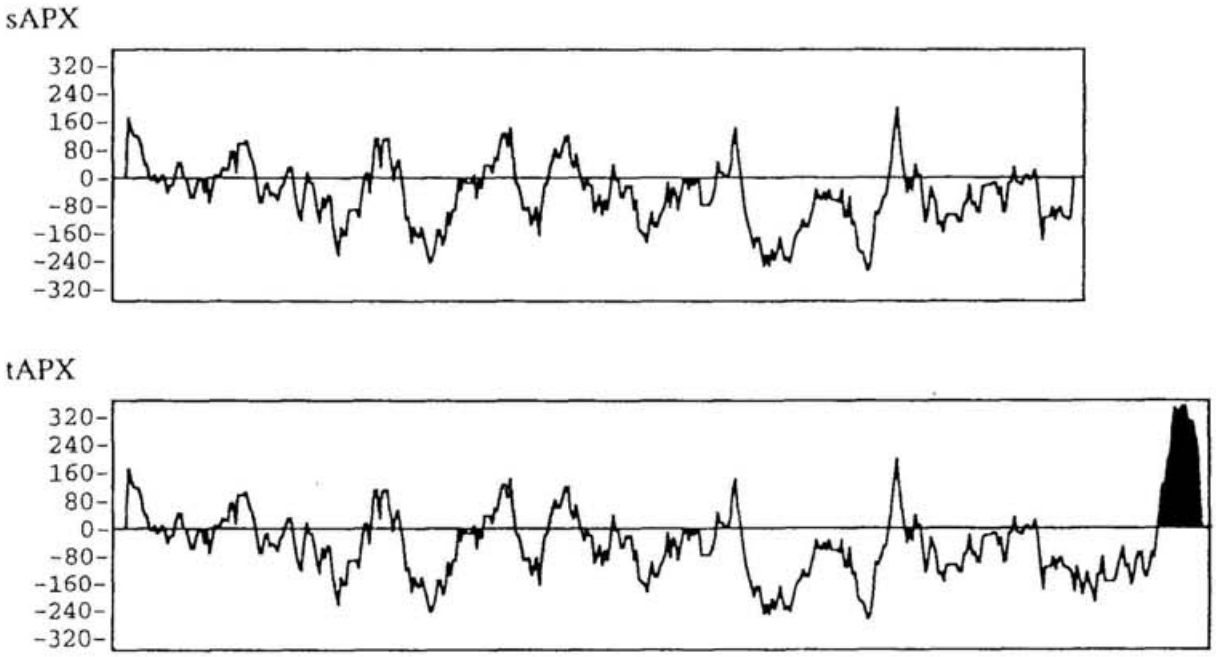
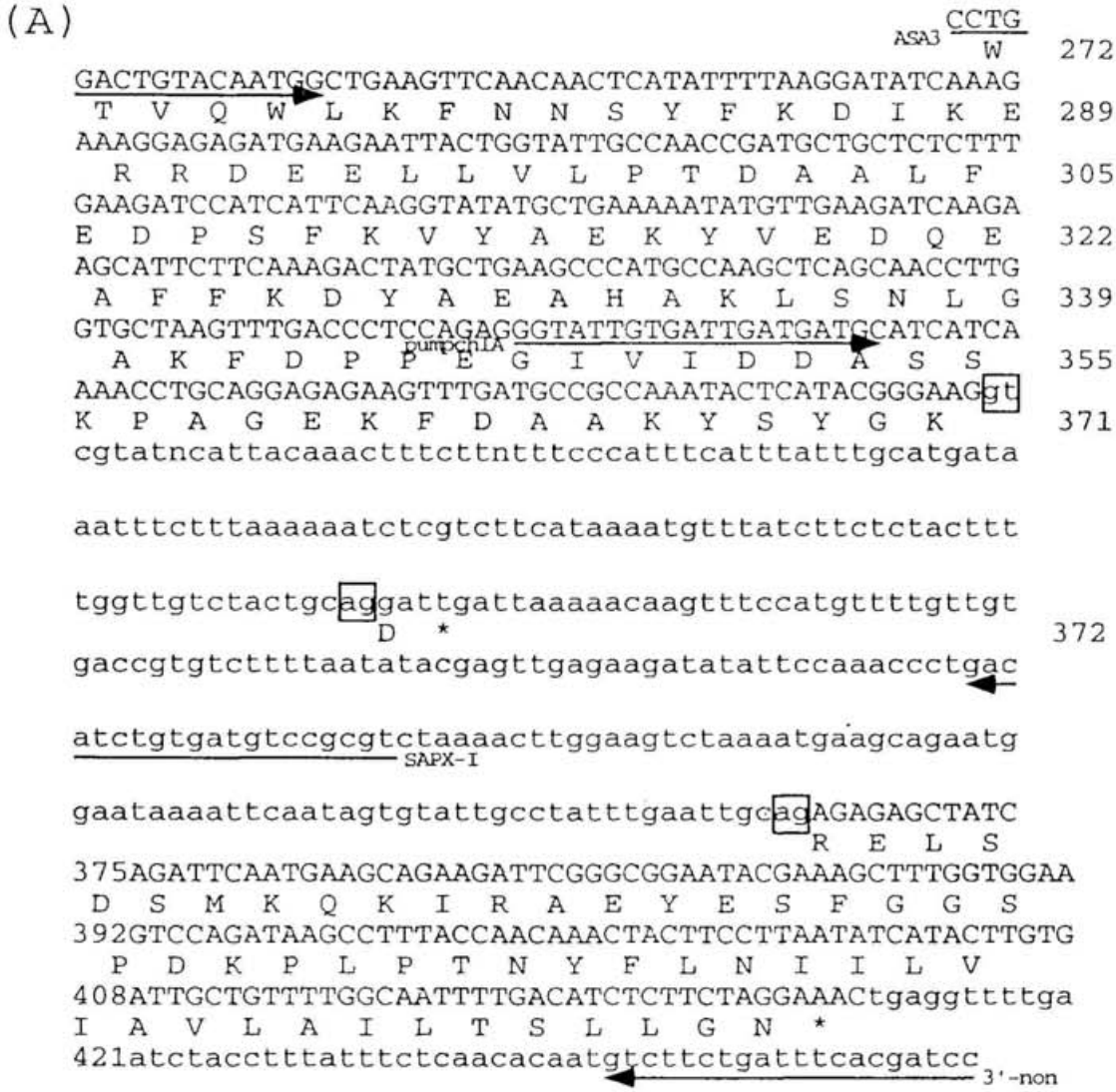


Figure 4-2. Comparison of sAPX and tAPX

(A) Comparison of the deduced amino acid sequences of the carboxy termini of sAPX and tAPX. (B) Comparison of the nucleotide sequences encoding the carboxy termini of sAPX and tAPX. (C) Hydropathy profiles of sAPX and tAPX. Positive values indicate hydrophobic regions. A putative membrane-spannin domain in tAPX is filled.

(A)


ASA3
CCTG
W
272
GACTGTACAATGGCTGAAGTTCAACAACTCATATTTTAAGGATATCAAAG
T V Q W L K F N N S Y F K D I K E
289
AAAGGAGAGATGAAGAATTACTGGTATTGCCAACCGATGCTGCTCTCTTT
R R D E E L L V L P T D A A L F
305
GAAGATCCATCATTCAAGGTATATGCTGAAAAATATGTTGAAGATCAAGA
E D P S F K V Y A E K Y V E D Q E
322
AGCATTCTTCAAAGACTATGCTGAAGCCCATGCCAAGCTCAGCAACCTTG
A F F K D Y A E A H A K L S N L G
339
GTGCTAAGTTTGACCCTCCAGAGGGTATTGTGATTGATGATGCATCATCA
pumpchlA
A K F D P P E G I V I D D A S S
355
AAACCTGCAGGAGAGAAGTTTGATGCCGCCAAATACTCATACGGGAAGgt
K P A G E K F D A A K Y S Y G K
371
cgtatncattacaaactttcttntttcccatttcatttatttgcatgata
aatttctttaaaaaatctcgtcttcataaaatgtttatcttctctacttt
tggttgtctactgcagGgattgattaaaaacaagtttccatgttttgttgt
D *
372
gaccgtgtcttttaatatacgagttgagaagatatattccaaaccctgac
atctgtgatgtccgcgtctaaaacttggaagtctaaaatgaagcagaatg
SAPX-I
gaataaaattcaatagtgtattgcctatttgaattgcagAGAGAGCTATC
R E L S
375AGATTCAATGAAGCAGAAGATTCGGGCGGAATACGAAAGCTTTGGTGGAA
D S M K Q K I R A E Y E S F G G S
392GTCCAGATAAGCCTTTACCAACAAACTACTTCCTTAATATCATACTTGTG
P D K P L P T N Y F L N I I L V
408ATTGCTGTTTTGGCAATTTTGACATCTCTTCTAGGAAACtgaggttttga
I A V L A I L T S L L G N *
421atctacctttatttctcaacacaatgtcttctgatttcacgatcc
3'-non

(B)

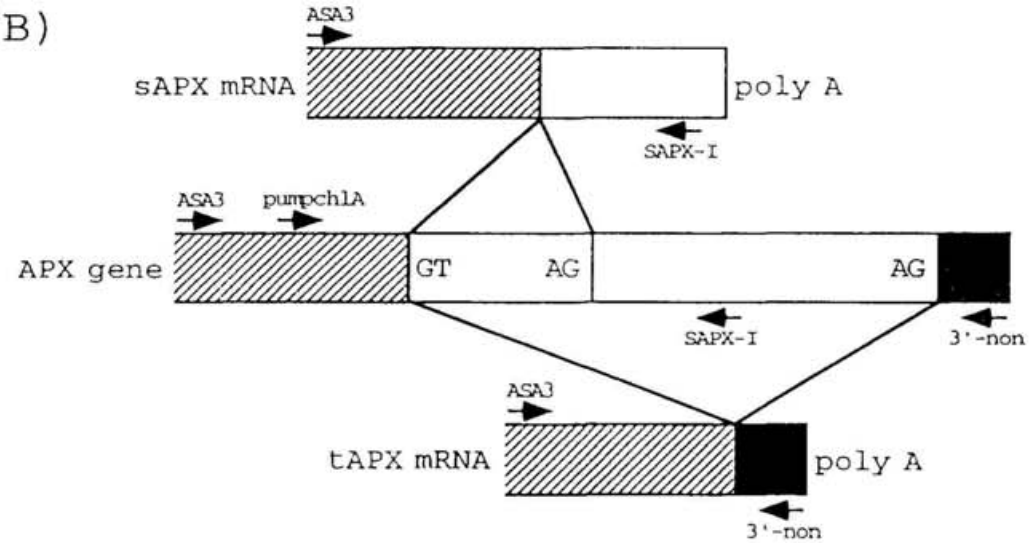

ASA3
sAPX mRNA
poly A
SAPX-I
ASA3
pumpchlA
APX gene
GT
AG
AG
SAPX-I
3'-non
ASA3
tAPX mRNA
poly A
3'-non

Figure 4-3 (A) The nucleotide sequence coding for the carboxy terminal region of the pumpkin chloroplastic APX gene is shown. The nucleotide sequence of the exon is shown in uppercase letters and the intron and 3'-noncoding region are shown in lowercase letters. The deduced amino acid sequence is presented in the single-letter code under the nucleotide sequence with numberings on the right. Asterisks indicate the stop codons and boxes represent the consensus sequences found in the beginning (GT) and the end (AG) of an intron. PCR primers, ASA3, pumpchl A, SAPX-I and 3'-non, are underlined with an arrowhead indicating the polarity. (B) Schematic representation of mRNAs and the gene. The hatched and filled boxes correspond to exons and the open boxes correspond to introns. Primers for PCR and the consensus sequences corresponding to the splice donor and acceptor sites are shown.

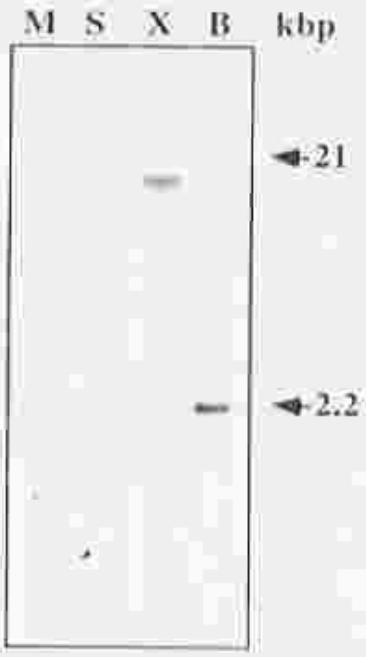


Figure 4-4. Southern blot analysis of pumpkin genomic DNA.

Genomic DNA (10 μg each) was digested with the restriction endonucleases indicated, separated on a 0.8% agarose gel, transferred to a Zeta-Probe blotting membrane (BIO-RAD) and probed with a [^{32}P]-labeled pumpkin sAPX cDNA fragment. The size of DNA fragments are shown in bp on the right. M, *Mlu*I ; S, *Sma*I; X, *Xho*I; B, *Bam*HI.

RT-PCR analysis

An RT-PCR was performed to monitor the presence of two kinds of mRNA as templates of first-strand cDNAs synthesized from mRNAs of 5-day dark- and 4-day light-grown cotyledons. The 5'-common sense primer of sAPX and tAPX (AsA3), and the 3'-specific antisense primers for tAPX (3'-non) and sAPX (SAPX-I) were used. Judging from the nucleotide sequences of sAPX (Figure 4-3, A) and tAPX (Yamaguchi et al., 1996), the combination of the AsA3 and 3'-non primers should give a 508-bp fragment and that of the AsA3 and SAPX-I primers should give a 406-bp fragment. These bands can be seen in lanes tAPX and sAPX of Figure 4-5, respectively. Figure 4-5 shows that each band is amplified in accordance with specific primers, suggesting that two kinds of mRNA are certainly expressed within pumpkin leaves.

Developmental changes in the relative amounts of sAPX and tAPX polypeptides in pumpkin cotyledons after germination

To obtain clues on expressions of sAPX and tAPX, developmental changes in the levels of sAPX and tAPX proteins in pumpkin cotyledons during germination and subsequent greening was investigated. The relative amounts of two kinds of chloroplastic APXs in total extracts from pumpkin cotyledons grown in the light and in the dark were determined by immunoblotting (top panels in Figure 4-6). The immunoblot was quantified with a densitometer and plotted (bottom panels in Figure 4-6). When seedlings were grown in darkness, the level of tAPX protein (right lower panel, closed circles) was hardly detected until the 5th day, whereas the level of sAPX protein (left lower panel, closed circles) increased gradually. The amounts of two proteins increased rapidly when 5-day-old seedlings were transferred to an illuminated area (lower panels, open circles). However, the different levels of induction of sAPX and tAPX in 7-day-old greening seedlings in the dark suggest that alternative splicing for sAPX and tAPX mRNAs in the cotyledons might be regulated by their developmental and environmental conditions such as light. Although this experiment with different extract preparations was performed several times, the similar developmental patterns were obtained in all cases.

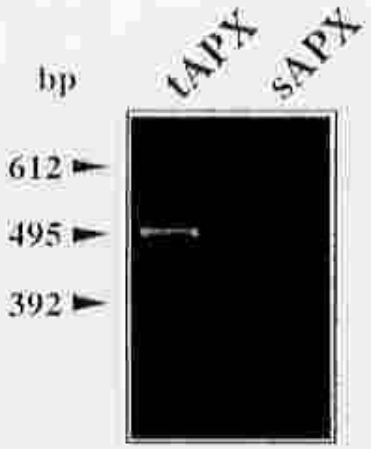


Figure 4-5. PCR amplification of truncated sAPX- and tAPX-specific mRNA.

RT-PCR was carried out using mRNAs prepared from 5-day dark- and 2-day light-grown cotyledons. The products were analyzed by electrophoresis on a 1% agarose gel and stained with ethidium bromide.

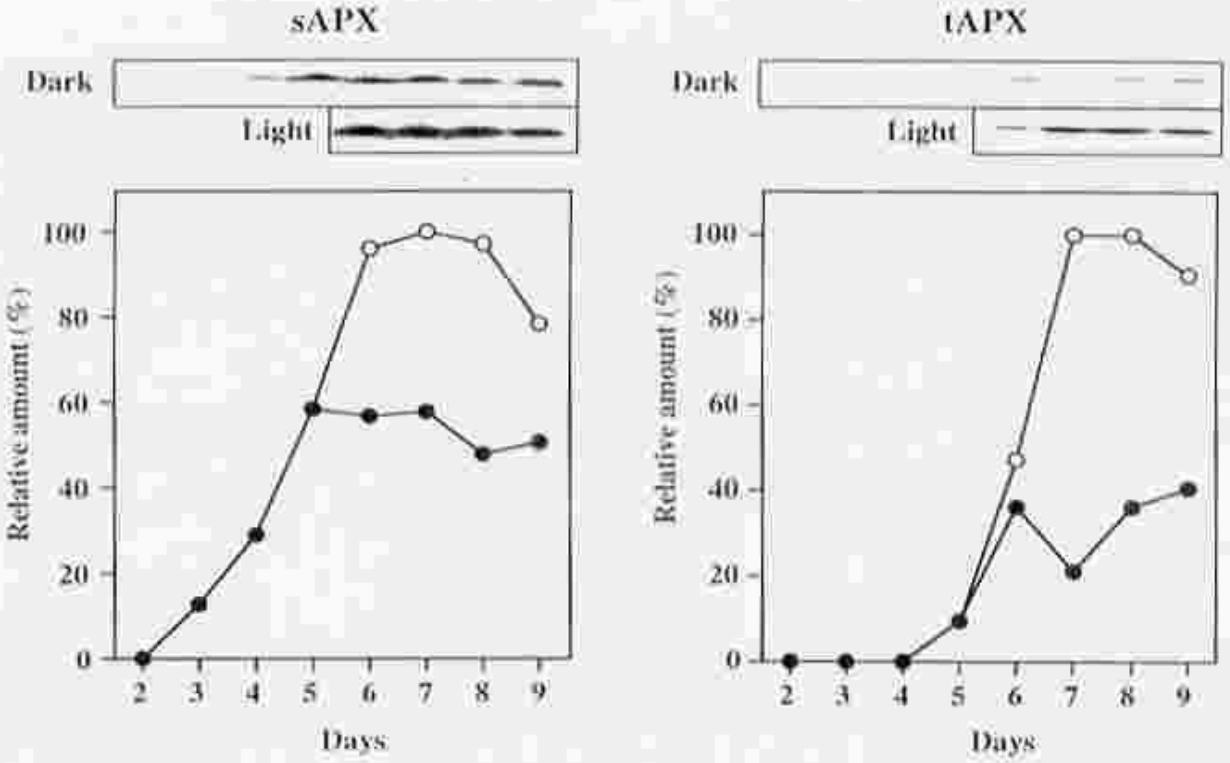


Figure 4-6. Developmental changes in the relative amounts of sAPX and tAPX polypeptides in pumpkin cotyledons after germination.

Total protein prepared from pumpkin cotyledons was subjected to SDS-PAGE, which was followed by immunodetection with antibodies against mbAPX (top panels). The relative amounts of the sAPX and tAPX polypeptides were determined densitometrically and plotted (bottom panels). (●) seedlings grown in darkness; (○) seedlings transferred to continuous illumination at the 5-day stage.

Tissue-specific expression of four APXs

To obtain further information about APXs, tissue specificity was investigated. Figure 4-7 shows the distribution of four APXs in different tissues. cAPX is the only APX that exists in all tissues although the molecular mass of cAPX in the petal is smaller than that in other tissues. mbAPX is exclusively detected in cotyledons and not in pollen and roots. With regard to chloroplastic APXs, much amounts of sAPX is in petals and dark-grown cotyledons although the amount in the petals is very low compared to that in the cotyledons. In addition, the low amount of sAPX is detected in the extracts of roots and pollen. The polypeptide of tAPX is present in only cotyledons and the amount increased after treatment with continuous light.

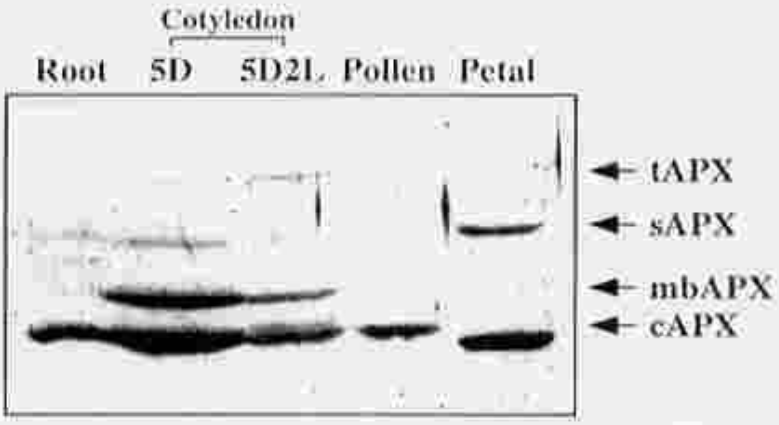


Figure 4-7. Tissue specific expression of four APXs

Total protein prepared from various organs was subjected to SDS-PAGE (root and cotyledon, 10μg each; pollen and petal, 80μg each), which was followed by immunodetection with antibodies against pumpkin mbAPX. 5D; 5-day dark-grown cotyledon; 5D2L, 5-day dark- and 2-day-light grown cotyledon. Arrows on the right represent the polypeptides for tAPX, sAPX, mbAPX and cAPX.

4.5. Discussion

Chloroplastic APXs have been purified and the molecular properties of purified enzymes have been characterized well (Chen and Asada, 1989; Chen et al., 1992; Miyake and Asada, 1992; Miyake et al., 1993). However, cDNA cloning of chloroplastic APXs has not been performed to date although the partial amino acids have been determined. Recently, Yamaguchi et al. (1996) reported the sequence of tAPX in pumpkin and Ishikawa et al. (1996) have cloned cDNAs for tAPX and sAPX in spinach. Nevertheless, information about chloroplastic APX remains to be so insufficient that cDNA cloning of pumpkin sAPX was carried out. From the nucleotide sequence analysis and the comparison with tAPX cDNA, it was sure that cloned cDNA encoded sAPX. This cDNA showed complete identity with tAPX cDNA except for the deletion of the domain encoding a putative membrane spanning region located in the carboxy domain of tAPX (Figure 4-2), indicating that alternative splicing leads to the synthesis of tAPX and sAPX mRNAs and that the deletion of the putative membrane spanning region gives the localization of sAPX protein in the stroma. The determination of genomic structure and the Southern blot analysis revealed that there are one donor site and two acceptor sites in one chloroplastic gene (Figure 4-3 and 4-4). Based on these data, it was assumed that the way to use each acceptor site affected the production of each mRNA. Namely, if the distal acceptor site from the donor site is used, the longer mRNA is produced, leading to the translation to tAPX protein that contains the putative membrane spanning region. However, the use of proximal acceptor site produces the shorter mRNA that is translated to sAPX protein. In this case, sAPX protein must exist in the stroma because of the lack the thylakoid-membrane spanning region. Two mRNAs corresponding to each chloroplastic APX are certainly transcribed in pumpkin green cotyledons (Figure 4-5). The immunoblot analysis of the changes during development after germination using antibodies of pumpkin mbAPX, which recognize four APX isoenzymes with molecular masses of 38, 34, 31 and 28 kDa (Yamaguchi et al., 1996), demonstrated that the accumulation of each APX protein was regulated by light (Figure 4-6). In the darkness, only sAPX exists since thylakoid membranes scarcely develop in etioplasts. When light

induces the conversion from etioplasts to chloroplasts, not only the accumulation of sAPX but also that of tAPX are induced such as other chloroplastic enzymes (Gilmartin et al., 1990). Because thylakoid membranes develop to acquire the ability of photosynthesis. These data indicate the presence of alternative splicing that is controlled by light. With respect to chloroplastic APXs, especially tAPX, biochemical properties have been characterized but the mechanism of gene expression remains to be clarified. To obtain clues, tissue-specific expression of four APXs was investigated (Figure 4-7). All tested tissues contains cAPX. It has been reported that mRNA for cAPX is induced by environmental stimuli such as drought and heat stress (Mittler and Zilinskas, 1992; 1994), and the rises of cAPX activity and amount of cAPX mRNA during fruit ripening was reported in bell pepper (Schantz et al., 1995), although the function remains to be obscure. The fact that all tested tissues have cAPX implies the presence of the important physiological role in the cytosol. cAPX might scavenge the stress-induced hydrogen peroxide. mbAPX is present in cotyledons and not in roots, pollen and petals. According to Yamaguchi et al. (1995), mbAPX has a role of the reduction of hydrogen peroxide produced by β-oxidation in glyoxysomes of dark-grown cotyledons and by photorespiration in leaf peroxisomes of light-grown cotyledons. For the present, root tissues are not considered to have β-oxidation and the function of photorespiration. If unspecialized microbodies in roots do not produce large amounts of hydrogen peroxide, mbAPX might not necessarily express. Detailed analyses about microbodies in petals and pollen have not been reported but De Bellis et al. (1991) reported the presence of leaf peroxisomal enzyme in the petal at the beginning of senescence, showing that microbodies in petals are leaf peroxisomes and not unspecialized microbodies. Then mbAPX should be present in petals. One interpretation is that the components and function of the leaf peroxisomal membrane in petals are different from those of cotyledons. With regard to chloroplastic tAPX, it exists in cotyledons. This is in good agreement with the data in Figure 4-6. Other tissues do not have tAPX because of the immature of the thylakoid membranes. sAPX is detected in most tissues although the amounts depend on each tissue. It is of interest to note that only sAPX, but not tAPX, is

present in petal tissues, indicating that the specific production of sAPX mRNA by alternative splicing or the specific degradation of tAPX. The results mentioned above are the first report of the analyses of about expression of chloroplastic APXs. Chloroplastic APX homologues have been found very recently in *Arabidopsis* (Jespersen et al., 1997). They reported that the transit sequences between sAPX and tAPX proteins were different, concluding that *Arabidopsis* clearly has two different genes for chloroplastic APXs. In pumpkin, chloroplastic APX exists as a single gene (Figure 4-4). From the analysis of nucleotide and deduced amino acid sequences, Jespersen et al. (1997) presumed that the chloroplast branch has apparently diverged into stromal and thylakoid-bound types in *Arabidopsis*.

Chapter 5
General discussion

Summary

Alternative splicing, which produces some variants from single genes, has been considered as one of the post-transcriptional devices, and it has been reported that alternative splicing is involved in the synthesis of some proteins in eukaryotes. Analysis on alternative splicing has been mainly made progress in mammals. However, recent studies revealed that alternative splicing is related to the maturation of some pre-mRNAs in higher plants. Two proteins, which I presented in this study, are also regulated by alternative splicing. In both cases, alternative splicing results in two variants whose subcellular or suborganellar localizations are different.

In Chapter 2, I showed the presence of two HPR proteins in pumpkin cells. HPR functions as a member of photorespiration and has been thought to be localized in leaf peroxisomes in the photosynthetic tissues. Two kinds of HPR cDNA were cloned and determined their nucleotide sequences (Hayashi et al., 1996) (Figure 1-3), suggesting that two HPR mRNAs might be produced by alternative splicing. In addition, it has been anticipated, based on deduced amino acid sequences, that HPR1 and HPR2 proteins are localized in leaf peroxisomes and in the cytosol, respectively, because HPR1 protein, but not HPR2 protein, contains a targeting signal to microbodies at the carboxy terminus. The immunoblot analysis and the subcellular fractionation experiment showed the presence of two electrophoretically similar but separable polypeptides (Figure 2-4) and that high activity of HPR in the cytosolic fraction was detected compared to that in the leaf peroxisomal fraction. Indirect immunofluorescent microscopy certainly revealed two different localizations of the immunofluorescence, namely, in leaf peroxisomes and the cytosol (Figure 2-6). Moreover, the analysis using transgenic plants confirmed the different subcellular localizations of the two HPR proteins (Figure 2-8 and 2-9). These findings seem to originate from the presence of the targeting signal to microbodies that is produced by alternative splicing. Almost the same amounts of two polypeptides corresponding to HPR1 and HPR2 proteins were present in the extracts from dark-grown cotyledons. When the cotyledons were transferred to continuous light, the amounts of HPR mRNAs and polypeptides, especially HPR2, increased markedly (Figure 2-2 and 2-

4). This result is in good agreement with the analysis by RT-PCR (Figure 2-3). These findings showed that light seems to shift the pattern for splicing of HPR to greater production of HPR2 mRNA. At present, the presence of alternative splicing that is regulated by light has not been reported in any organisms. To obtain further information, tissue specificity and temperature effect were investigated. As shown in Figure 2-10, the two polypeptides corresponding to HPR proteins were present in light-grown cotyledons, leaves, whole flowers and petals, whereas not in roots. Moreover, it is important to note that only HPR2 protein, but not HPR1 protein, was detected in the extracts from the stamen. The stamen is the only tissue that has one kind of HPR protein as far as I have investigated. The mechanisms that produces only HPR2 protein or that is involved in the specific degradation of HPR1 protein might exist and function in stamen cells. This result implies that the stamen might have the possibility of the good control tissue for analysis of alternative splicing of HPR. The shift of the temperature from 25°C to 35°C did not affect the expression of HPR. Moreover, the amounts of HPR proteins in the petals and the stamen were decreased with their senescence *in vivo*. These results are similar to that in cotyledons (Mano et al., 1996).

In Chapter 3, I characterized *Arabidopsis* HPR to obtain clues about information of HPR. Although the HPR gene in *Arabidopsis* exists as a single copy, as it does in pumpkin and cucumber (Figure 3-4), the genomic structure in *Arabidopsis* is different from that in pumpkin. That is, the HPR gene in pumpkin has the presence of two pairs of GT-AG doublets within the intron, whereas *Arabidopsis* HPR gene contains only one GT-AG doublet (Figure 3-3), suggesting that alternative splicing might not occur against our expectations. It is sure that all EST clones have the targeting signal to microbodies at the carboxy termini (Figure 3-2) and that only one polypeptide was recognized by the HPR-specific antibody (Figure 3-6). Therefore, I concluded that *Arabidopsis* has one HPR protein with a targeting signal to microbodies. Then how many kinds of HPR protein do other plants have? To address this question, I tried to determine by the immunoblot analysis whether the two HPR proteins exist in various plants (Figure 3-7). In addition to pumpkin, two electrophoretically similar bands are present in the extracts

from cucumber but not in other plants or other ecotypes of *Arabidopsis*. Based on the genomic structure, I have expected that two HPR proteins are probably present in cucumber. Two polypeptides would be probably localized in the leaf peroxisomes and the cytosol. These results imply that the differences in the gene structure and the regulation of gene expression of HPR cause in species-specific manner in higher plants.

In Chapter 4, I showed the characterization of chloroplastic APXs as the second example that is regulated by alternative splicing. I succeeded in cloning of pumpkin sAPX cDNA and determined its nucleotide sequence (Figure 4-1). The nucleotide and deduced amino acid sequences showed the complete identity except the carboxy terminal domain (Figure 4-2), suggesting the involvement of alternative splicing to the production of sAPX and tAPX mRNAs. That is, although alternatively spliced sAPX protein contains the transit peptide at the amino terminal end as well as tAPX protein does, the putative thylakoid-membrane spanning region located at the carboxy domain of tAPX protein seems to be eliminated by alternative splicing (Figure 4-3). I investigated the developmental changes of two chloroplastic APX proteins (Figure 4-6). When cotyledons were kept in darkness, the amount of sAPX protein increased gradually and reach to the maximum at the 5-day stage, whereas that of tAPX protein was kept at the low level for a few days and then increased slightly. In the case that cotyledons were transferred to continuous light, the amounts of both proteins were induced markedly. From the densitometrically estimation of the amounts of two chloroplastic APX proteins, light shifted the pattern for splicing of chloroplastic APXs pre-mRNA from the accumulation of sAPX mRNA to the almost accumulations of sAPX and tAPX mRNAs, resulting in the remarkable change of the ratio of sAPX to tAPX. These results showed that alternative splicing that is involved in the production of chloroplastic APXs is regulated developmentally and by light. Analysis of tissue specificity revealed the presence of sAPX protein, but not tAPX protein, in the petals (Figure 4-7).

From the results of these two examples, it is demonstrated the presence of alternative splicing that produces variants whose subcellular or suborganellar localizations

are different. Moreover, it is revealed that this alternative splicing showed the light-dependent and the tissue-specific manner.

General discussion

The acquirement of complex biological system has been accompanied by increasing genomic complexity to generate multiple variants that are adapted to function in a particular cell type, developmental stage or physiological state. To produce genomic complexity leading to protein diversity, three main mechanisms have evolved: gene duplication, gene rearrangement and alternative splicing. Of the three, alternative splicing has a lot of advantages and few of the limitations of gene duplication and gene rearrangement. Gene duplication is the most costly in genetic term because each isoform requires the duplication of the entire genetic sequence. However, the abundance of multigene families demonstrates the evolutionary success of this process. Gene rearrangement is an efficient mechanism for the generation of proteins diversity from single genes. The immunoglobulin genes contain intragenic duplications that correspond to the variable domains of the protein. In this case, the combination of a limited set of V, D and J exons can produce an almost unlimited number of different antibodies. Yet surprisingly, this mechanism seems to be restricted to the immune system. Perhaps DNA rearrangement can be used only by cells in the process of terminal differentiation because it irreversibly changes the genetic content of the cell. On the contrary, alternative splicing does not change the genetic content of the cell nor is irreversible in genetic terms. In addition, splicing pathway need not be discarded in order to use different ones, making alternative splicing particularly suited to generate protein diversity.

The differences of structural characteristics of HPR and chloroplastic APXs between pumpkin and another plants provide the basis for a discussion of the evolution of HPR and chloroplastic APXs. Spinach, pea and barley have the two HPR proteins with different subcellular localizations as does pumpkin, whereas only one HPR exists in leaf peroxisomes of *Arabidopsis*. However, the two HPR proteins of spinach, pea and barley

originated in different genes. In addition, there are at least two genes of chloroplastic APX in *Arabidopsis*, although pumpkin and spinach have only one gene. Judging from these results, alternative splicing of HPR and chloroplastic APXs is species-specific and it probably depends on the physiological necessities in each plant.

Alternative splicing utilizes different promoter, different splice sites and different poly(A) sites in order to produce multiple variants and it is important as a post-transcriptional level of gene regulation like the transcriptional level, because it affects protein localization, protein activity, production of novel protein and RNA stability and translational efficiency. With respect to different localizations, as described previously in Chapter 1.1.2, carnitine acetyltransferase (Corti et al., 1994), human interleukin-6 signal transducer gp130 (Diamant et al., 1997), CD44 (Yu and Toole, 1996), rat A3 adenosine receptor (Sajjadi et al., 1996) undergo alternative splicing, leading to the localizations of different parts. In these cases, ones are produced as membrane-bound proteins and the others are secreted because they are produced as soluble proteins. Interestingly, these soluble proteins appear to function as antagonists of their membrane-bound forms, inhibitors of self-stimulatory effects or mediators of various signals. In higher plants, the variants of the small subunit of AGPase (Thorbjørnsen et al., 1996), FCA proteins, a controlling flowering time (Macknight et al., 1997) and ARK1 protein (Tobias and Nasrallah, 1996) show different localizations in cells. Of these, in the only case of the small subunit of AGPase, the function of alternatively spliced variant has been clarified (Denyer et al., 1996; Thorbjørnsen et al., 1996).

Both chloroplastic APXs, of two proteins that I analyzed in this study, have been demonstrated to fulfill as a function of the scavenger of hydrogen peroxide in spinach chloroplasts (Miyake et al., 1993). However, as discussed in Chapter 2.5, the physiological role of cytosolic HPR remains to be determined. To obtain clues on alternative splicing including the function of the cytosolic HPR, I attempted to use *Arabidopsis thaliana* because of the possibility of making transgenic plants. As the first step, the molecular cloning and characterization of *Arabidopsis* HPR were performed. As demonstrated in Chapter 3, *Arabidopsis* (ecotype Columbia) has only one HPR with the

peroxisomal targeting signal as opposed to our expectation. In addition, the analysis of other plants and another ecotypes of *Arabidopsis* also revealed the presence of one kind of HPR protein except cucumber. These data suggest that plants might be able to survive without the cytosolic HPR. However, only HPR2 is present in the stamen and the amount of HPR2 protein is more than that of HPR1 protein in the cotyledons of pumpkin and cucumber. Moreover, light-induction causes the much production of HPR2 mRNA in pumpkin. These results indicate the presence of an unidentified role in only *Cucurbita* species.

Although the mechanism on general and alternative splicing have not been completely clarified, some components consisting of the spliceosome and elements necessary for splicing have been identified (Mount, 1982; Madhani and Guthrie, 1994). The number of novel components are expanding even in the best characterized mechanism of the sex determination of *Drosophila*. A family of proteins with arginine- and serine-rich domains, SR protein, has recently come into the limelight of studies on the mechanisms of general and alternative splicing. SR proteins are phosphorylated *in vivo* (Roth et al., 1990; Yitzhaki et al., 1996), although the role of phosphorylation is obscure. Their phosphorylation-dephosphorylation might be required for the assembly with pre-RNAs or other proteins. Implicated in an ever increasing variety of function, these SR proteins act as driving forces during spliceosomes assembly and also play decisive roles in the site selection of alternative splicing (Valcárcel and Green, 1996). For example, ASF/SF2, which is a kind of SR proteins, functions as a general splicing factor, whereas it can also switch between alternative splice sites when it is present in excess, indicating the importance of the concentration of splicing factors. Gallego et al. (1997) certainly reported that the ratio of ASF/SF2 to another member of the SR family, SC35, affected the exon 6A splicing in β-tropomyosin pre-mRNA. In addition, SR proteins modulate alternative splicing of another SR proteins and their own pre-mRNAs (Screaton et al., 1995; Jumaa and Nielsen, 1997). The expression of individual SR proteins shows cell-type-specific, developmental stage-specific or tissue-specific manners. Sxl protein, as introduced in Chapter 1.1.3, is also the SR protein and it promotes the synthesis of its

own female-specific mRNA as well as tra pre-mRNA (Sakamoto et al., 1992; Wang et al., 1997). It is probably expected that the number of SR proteins would expand in the future.

In mammals, *in vitro* splicing assays using HeLa cell nuclear extract has been established (Dignam et al., 1983), contributing to the analysis of the identification of components, *cis* regulatory elements and their functions. Lazar et al. (1995) and Lopato et al. (1996) demonstrated that ASF/SF2 homologues in *Arabidopsis*, carrot and tobacco could function in splice site switching of the legumin pre-mRNA and human β-globin pre-mRNA in this nuclear extracts, although the splicing activities were due to species-specific differences in the SR proteins. This data indicates that plant SR proteins are active in a heterologous alternative splicing assay system. Probably, this splicing system *in vitro* will be useful in the analysis of HPR and chloroplastic pre-mRNAs. I am now trying to establish the splicing assay system *in vitro* in plants using tobacco cultured cells. In addition, it is important to settle the splicing assay system *in vivo*, such as the particle bombardment, since the light-dependency is the specific feature to plants and the splicing assay system using HeLa cell nuclear extract might not be useful. The establishment of the splicing assay system, the identification of some components and *cis*-elements will provide the essential new insights on the mechanisms of alternative splicing.

References

Arsura, M., Deshpande, A., Hann, S. R. and Sonenshein, G. E. (1995). *Mol. Cell. Biol.*, **15**, 6702-6709

Baker, B. S. (1989). *Nature*, **340**, 521-524

Bechtold, N., Ellis, J. and Pelletier, G. (1993). *C. R. Acad. Sci. Paris, Life Sciences*, **316**, 1194-1199

Bell, L. R., Maine, E. M., Schedl, P. and Cline, T. W. (1988). *Cell*, **55**, 1037-1046

Bertoni, G. P. and Becker, W. M. (1996). *Plant Physiol.*, **112**, 599-605

Blattner, J., Swinkels, B., Dörsam, H., Prospero, T. and Subramani, S. (1992). *J. Cell Biol.*, **119**, 1129-1136

Bunkelmann, J. R. and Trelease, R. N. (1996). *Plant Physiol.*, **110**, 589-598

Chandler, S. D., Mayeda, A., Yeakley, J. M., Krainer, A. R. and Fu, X.-D. (1996). *Proc. Natl. Acad. Sci. U.S.A*, **94**, 3596-3601

Chen, G.-X. and Asada, K. (1989). *Plant Cell Physiol.*, **30**, 987-998

Chen, G.-X., Sano, S. and Asada, K. (1992). *Plant Cell Physiol.*, **33**, 109-116

Chistoserdova, L. V. and Lidstrom, M. E. (1991). *J. Bacteriol.*, **173**, 7228-7232

Chistoserdova, L. V. and Lidstrom, M. E. (1994). *J. Bacteriol.*, **176**, 1957-1968

Comai, L., Dietrich, R. A., Maslyar, D. J., Baden, C. S. and Harada, J. J. (1989). *Plant Cell*, **1**, 293-300

Corti, O., Didonato, S. and Finocchiaro, G. (1994). *Biochem. J.*, **303**, 37-41

De Bellis, L. and Nishimura, M. (1991). *Plant Cell Physiol.*, **32**, 555-561

De Bellis, L., Tsugeki, R. and Nishimura, M. (1991). *Plant Cell Physiol.*, **32**, 1227–1235

Denyer, K., Dunlap, F., Thorbjørnsen, T., Keeling, P. and Smith, A. M. (1996). *Plant Physiol.*, **112**, 779-785

Diamant, M., Rieneck, K., Mechti, N., Zhang, X.-G., Svenson, M., Bendtzen, K. and Klein, B. (1997). *FEBS Lett.*, **412**, 379-384

Dignam, J. D., Lebovitz, R. M. and Roeder, R. G. (1983). *Nucleic Acids Res.*, **11**, 1475-1489

Duncan, P. I., Howell, B. W., Marius, R. M., Drmanic, S., Douville, E. M. J. and Bell, J. C. (1995). *J. Biol. Chem.*, **270**, 21524-21531

Dunstan, R. M., Anthony, C. and Drabble, W. T. (1972). *Biochem. J.*, 128, 107-115

Fransen, M., Brees, C., Baumgart, E., Vanhooren, J. C. T., Baes, M., Mannaerts, G. P. and Veldhoven, P. P. V. (1995). *J. Biol. Chem.*, **270**, 7731-7736

Gallego, M. E., Gattoni, R., Stévenin, J., Marie, J. and Expert-Bezançon, A. (1997). *EMBO J.*, **16**, 1772-1784

Gilmartin, P. M., Sarokin, L., Memelink, J. and Chua, N.-H. (1990). *Plant Cell*, **2**, 369-378

Givan, C. V. and Kleczkowski, L. A. (1992). *Plant Physiol.*, **100**, 552-556

Gontarek, R. R. and Derse, D. (1996). *Mol. Cell. Biol.*, **16**, 2325-2331

Gould, S. J., Keller, G.-A., Hosken, N., Wilkinson, J. and Subramani, S. (1989). *J. Cell Biol.*, **108**, 1657-1664

Gould, S. J., Keller, G.-A., Schneider, M., Howell, S. H., Garrard, L. J., Goodman, J. M., Distel, B., Tabak, H. and Subramani, S. (1990). *EMBO J.*, **9**, 85-90

Gould, S. J., Keller, G.-A. and Subramani, S. (1987). *J. Cell Biol.*, **105**, 2323-2931

Gould, S. J., Keller, G.-A. and Subramani, S. (1988). *J. Cell Biol.*, **107**, 897-905

Graham, I. A., Smith, L. M., Brown, J. W. S., Leaver, C. J. and Smith, S. M. (1989). *Plant Mol. Biol.*, **13**, 673-684

Greenler, J. M., Sloan, J. S., Schwartz, B. W. and Becker, W. M. (1989). *Plant Mol. Biol.*, **13**, 139-150

Görlach, J., Raesecke, H.-R., Abel, G., Wehrli, R., Amrhein, N. and Schmid, J. (1995). *Plant J.*, **8**, 451-456

Han, P., Zhu, X. and Michaeli, T. (1997). *J. Biol. Chem.*, **272**, 16152-16157

Hayashi, M., Aoki, M., Kondo, M. and Nishimura, M. (1997). *Plant Cell Physiol.*, **38**, 759-768

Hayashi, M., Tsugeki, R., Kondo, M., Mori, H. and Nishimura, M. (1996). *Plant Mol. Biol.*, **30**, 183-189

Hoshijima, K., Inoue, K., Higuchi, I., Sakamoto, H. and Simura, Y. (1991). *Science*, **252**, 833-836

Huang, S. and Spector, D. L. (1996). *J. Cell Biol.*, **133**, 719-732

Husic, D. W. and Tolbert, N. E. (1987). *Archi. Biochem. Biophys.*, **252**, 396-408

Inoue, K., Hoshijima, K., Higuchi, I., Sakamoto, H. and Shimura, Y. (1992). *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 8092-8096

Inoue, K., Hoshijima, K., Sakamoto, H. and Shimura, Y. (1990). *Nature*, **344**, 461-463

Ishikawa, T., Sakai, K., Yoshimura, K., Takeda, T. and Shigeoka, S. (1996). *FEBS Lett.*, **384**, 289-293

Jespersen, H. M., Kjærsgård, I. V. H., Østergaard, L. and Welinder, K. G. (1997). *Biochem. J.*, **326**, 305-310

Jumaa, H. and Nielsen, P. J. (1997). *EMBO J.*, **16**, 5077-5085

Karlin-Neumann, G. A. and Tobin, E. M. (1986). *EMBO J.*, **5**, 9-13

Kato, A., Hayashi, M., Mori, H. and Nishimura, M. (1995). *Plant Mol. Biol.*, **27**, 377-390

Kato, A., Hayashi, M., Takeuchi, Y. and Nishimura, M. (1996). *Plant Mol. Biol.*, **31**, 843-852

Kato, A., Takeda-Yoshikawa, Y., Hayashi, M., Kondo, M., Ikuko, H.-N. and Nishimura, M. (1998). *Plant Cell Physiol.*, in press,

Kleczkowski, L. A. and Edwards, G. E. (1989). *Plant Physiol.*, **91**, 278-286

Kleczkowski, L. A., Givan, C. V., Hodgson, J. M. and Randall, D. D. (1988). *Plant Physiol.*, **88**, 1182-1185

Kleczkowski, L. A. and Randall, D. D. (1988). *Biochem. J.*, **250**, 145-152

Kohn, L. D. and Jakoby, W. B. (1968). *J. Biol. Chem.*, **243**, 2494-2499

Kopriva, S., Chu, C.-c. and Bauwe, H. (1996). *Plant J.*, **10**, 369-373

Koshiba, T. (1993). *Plant Cell Physiol.*, **34**, 713-721

Laemmli, U. K. (1970). *Nature*, **227**, 680-685

Lazar, G., Schaal, T., Maniatis, T. and Goodman, H. M. (1995). *Proc. Natl. Acad. Sci. U.S.A*, **92**, 7672-7676

Leij, I. V. D., Franse, M. M., Elgersma, Y., Distel, B. and Tabak, H. F. (1993). *Proc. Natl. Acad. Sci. U.S.A*, **90**, 11782-11786

Lievens, P. M. J., Tufarelli, C., Donady, J. J., Stagg, A. and Neufeld, E. J. (1997). *Gene*, **197**, 73-81

Lopato, S., Mayeda, A., Krainer, A. R. and Barta, A. (1996). *Proc. Natl. Acad. Sci. U.S.A*, **93**, 3074-9079

Lopato, S., Waigmann, E. and Barta, A. (1996). *Plant Cell*, **8**, 2255-2264

Lui, V. C. H., Ng, L. J., Sat, E. W. Y., Nicholls, J. and Cheah, K. S. E. (1996). *J. Biol. Chem.*, **271**, 16945-16951

Lumbreras, V., Campos, N. and Boronat, A. (1995). *Plant J.*, **8**, 541-549

Macknight, R., Bancroft, I., Page, T., Lister, C., Schmidt, R., Love, K., Westphal, L., Murphy, G., Sherson, S., Cobbett, C. and Dean, C. (1997). *Cell*, **89**, 737-745

Madhani, H. D. and Guthrie, C. (1994). *Annu. Rev. Genet.*, **28**, 1-26

Mano, S., Hayashi, M., Kondo, M. and Nishimura, M. (1996). *Plant Cell Physiol.*, **37**, 941-948

Mano, S., Hayashi, M., Kondo, M. and Nishimura, M. (1997). *Plant Cell Physiol.*, **38**, 449-455

Marchuk, D., Drumm, M., Saulino, A. and Collins, F. S. (1990). *Nucleic Acids Res.*, **19**, 1154-1154

Marzioch, M., Erdmann, R., Veenhuis, M. and Kunau, W.-H. (1994). *EMBO J.*, **13**, 4908-4918

Matsuoka, K. and Nakamura, K. (1991). *Proc. Natl. Acad. Sci. U.S.A*, **88**, 834-838

McCollum, D., Monosov, E. and Subramani, S. (1993). *J. Cell Biol.*, **121**, 761-774

McKeown, M. (1992). *Annu. Rev. Cell Biol.*, **8**, 133-155

Mittler, R. and Zilinskas, B. A. (1992). *J. Biol. Chem.*, **267**, 21802-21807

Mittler, R. and Zilinskas, B. A. (1994). *Plant J.*, **5**, 397-405

Mittler, R. and Zilinski, B. A. (1991). *FEBS Lett.*, **289**, 257-259

Miyake, C. and Asada, K. (1992). *Plant Cell Physiol.*, **33**, 541-553

Miyake, C., Cao, W.-H. and Asada, K. (1993). *Plant Cell Physiol.*, **34**, 881-889

Miyatake, R., Furukawa, A., Matsushita, M., Iwahashi, K., Nakamura, K., Ichikawa, Y. and Suwaki, H. (1996). *FEBS Lett.*, **395**, 123-126

Mori, H. and Nishimura, M. (1989). *FEBS Lett.*, **244**, 163–166

Mori, H., Takeda-Yoshikawa, Y., Hara-Nishimura, I. and Nishimura, M. (1991). *Eur. J. Biochem.*, **197**, 331-336

Mount, S. M. (1982). *Nucleic Acids Res.*, **10**, 459-472

Murray, A. J. S., Blackwell, R. D. and Lea, P. J. (1989). *Plant Physiol.*, **91**, 395-400

Murray, M. G. and Thompson, W. F. (1980). *Nucleic Acids Res.*, **8**, 4321-4325

Nakamura, K. and Matsuoka, K. (1993). *Plant Physiol.*, **101**, 1-5

Nishimura, M., Takeuchi, Y., Bellis, L. D. and Hara-Nishimura, I. (1993). *Protoplasma*, **175**, 131-137

Nishimura, M., Yamaguchi, J., Mori, H., Akazawa, T. and Yokota, S. (1986). *Plant Physiol.*, **80**, 313-316

Ohsima, Y. and Gotoh, Y. (1987). *J. Mol. Biol.*, **195**, 247-259

Padgett, R. A., Grabowski, P. J., Konarska, M. M., Seiler, S. and Sharp, P. A. (1986). *Annu. Rev. Biochem.*, **55**, 1119-1150

Paris, N. and Rogers, J. C. (1996). *Plant Physiol. Biochem.*, **34**, 223-227

Parker, R. and Guthrie, C. (1985). *Cell*, **41**, 107-118

Pelham, H. R. B. (1989). *Annu. Rev. Cell Biol.*, **5**, 1-23

Purdue, P. E. and Lazarow, P. B. (1994). *J. Biol. Chem.*, **269**, 30065-30068

Roise, D. and Schatz, G. (1988). *J. Biol. Chem.*, **263**, 4509-4511

Roth, M. B., Murphy, C. and Gall, J. G. (1990). *J. Cell Biol.*, **111**, 2217-2223

Sajjadi, F. G., Boyle, D. L., Domingo, R. C. and Firestein, G. S. (1996). *FEBS Lett.*, **382**, 125-129

Sakamoto, H., Inoue, K., Higuchi, I., Ono, Y. and Simura, Y. (1992). *Nucleic Acids Res.*, **20**, 5533-5540

Schantz, M.-L., Schreiber, H., Guillemaut, P. and Schantz, R. (1995). *FEBS Lett.*, **358**, 149-152

Schischmanoff, P. O., Yaswen, P., Parra, M. K., Lee, G., Chasis, J. A., Mohandas, N. and Conboy, J. G. (1997). *J. Biol. Chem.*, **272**, 10254-10259

Screaton, G. R., Cáceres, J. F., Mayeda, A., Bell, M. V., Plebanski, M., Jackson, D. G., Bell, J. I. and Krainer, A. R. (1995). *EMBO J.*, **14**, 4336-4349

Si, K., Das, K. and Maitra, U. (1996). *J. Biol. Chem.*, **271**, 16934-16938

Siliciano, P. G. and Guthrie, C. (1988). *Genes Dev.*, **2**, 1258-1267

Smith, C. W. J., Patton, J. G. and Nadal-Ginard, B. (1989). *Annu. Rev. Genet.*, **23**, 527-77

Snell, K. (1986). *Trends Biol. Sci.*, 11, 241-243

Sugiyama, T., Nishio, Y., Kishimoto, T. and Akira, S. (1996). *FEBS Lett.*, **381**, 191-194

Tacke, R., Chen, Y. and Manley, J. L. (1997). *Proc. Natl. Acad. Sci. U.S.A*, **94**, 1148-1153

Tamaoki, M., Tsugawa, H., Minami, E., Kayano, T., Yamamoto, N., Kano-Murakami, Y. and Matsuoka, M. (1995). *Plant J.*, 7, 927-938

Tarn, W.-Y. and Steitz, J. A. (1997). *Trends Biol. Sci.*, **22**, 132-137

Thorbjørnsen, T., Villand, P., Denyer, K., Olsen, O.-A. and Smith, A. M. (1996). *Plant J.*, **10**, 243-250

Thorbjørnsen, T., Villand, P., Kleczkowski, L. A. and Olsen, O.-A. (1996). *Biochem. J.*, **313**, 149-154

Titus, D. E. and Becker, W. M. (1985). *J. Cell Biol.*, **101**, 1288-1299

Tobias, C. M. and Nasrallah, J. B. (1996). *Plant J.*, **10**, 523-531

Tolbert, N. E., Oeser, A., Kisaki, T., Hageman, R. H. and Yamazaki, R. K. (1968). *J. Biol. Chem.*, **243**, 5179-5184

Tsugeki, R., Hara-Nishimura, I., Mori, H. and Nishimura, M. (1993). *Plant Cell Physiol.*, **34**, 51-57

Valcárcel, J. and Green, M. R. (1996). *Trends Biol. Sci.*, **21**, 296-301

Walker, W. H., Girardet, C. and Habener, J. F. (1996). *J. Biol. Chem.*, **271**, 20145-21050

Wang, J., Dong, Z. and Bell, L. R. (1997). *J. Biol. Chem.*, **272**, 22227-22235

Wetering, M. v. d., Castrop, J., Korinek, V. and Clevers, H. (1996). *Mol. Cell. Biol.*, **16**, 745-752

Wiemer, E. A. C., Nuttley, W. M., Bertolaet, B. L., Li, X., Francke, U., Wheelock, M. J., Anne, U. K., Johnson, K. R. and Subramani, S. (1995). *J. Cell Biol.*, **130**, 51-65

Yamaguchi, J. and Nishimura, M. (1984). *Plant Physiol.*, **262**, 261-267

Yamaguchi, K., Hayashi, M. and Nishimura, M. (1996). *Plant Cell Physiol.*, **37**, 405-409

Yamaguchi, K., Mori, H. and Nishimura, M. (1995). *Plant Cell Physiol.*, **36**, 1157-1162

Yamaguchi, K., Takeuchi, Y., Mori, H. and Nishimura, M. (1995). *Plant Cell Physiol.*, **36**, 455-464

Yang, T.-P., Agellon, L. B., Walsh, A., Breslow, J. L. and Tall, A. R. (1996). *J. Biol. Chem.*, **271**, 12603-12609

Yeakley, J. M., Morfin, J.-P., Rosenfeld, M. G. and Fu, X.-D. (1996). *Proc. Natl. Acad. Sci. U.S.A*, **93**, 7582-7587

Yitzhaki, S., Miriami, E., Sperling, R. and Sperling, J. (1996). *Proc. Natl. Acad. Sci. U.S.A*, **93**, 8830-8835

Yoshida, T., Yamaguchi, K., Hagishita, T., Mitsunaga, T., Miyata, A., Tanabe, T., Toh, H., Ohshiro, T., Shimao, M. and Izumi, Y. (1994). *Eur. J. Biochem.*, **223**, 727-732

Yu, Q. and Toole, B. P. (1996). *J. Biol. Chem.*, **271**, 20603-20607

Zahler, A. M., Lane, W. S., Stolk, J. A. and Roth, M. B. (1992). *Genes Dev.*, **6**, 837-847

Zhao, W. and Manley, J. L. (1996). *Mol. Cell. Biol.*, **16**, 2378-2386

Acknowledgments

I expresses my profound gratitude to Dr. Mikio Nishimura, Professor of Division of Cell Mechanisms, National Institute for Basic Biology; Department of Molecular Biomechanics, The Graduate University for Advanced Studies, for valuable discussions, support and encouragement throughout the work.

I thank very much to Dr. Makoto Hayashi, Dr. Ikuko Hara-Nishimura and Dr. Tomoo Shimada, Division of Cell Mechanism, National Institute for Basic Biology; Department of Molecular Biomechanics, The Graduate University for Advanced Studies, for their appropriate advises and kind guidances.

I wish to thank Dr. Kenzo Nakamura, Professor of School of Agriculture, Nagoya University for his kind allowance to use of Ti-vector, pMAT037 and provide BY-2, Dr. Yasuo Niwa, School of Food and Nutritional Sciences, University of Shizuoka for kindly providing cDNA of sGFP-TYG, Dr. N. -H. Chua, Rockefeller University for the antiserum against hydroxypyruvate reductase from spinach.

My research has been supported in part by the Fellowship for Japanese Junior Scientists funded by the Japan Society for the Promotion of Science (Apr. 1996-).

I feel grateful to all members of the Division of Cell Mechanisms, National Institute for Basic Biology, for their kind friendship and help. Lastly, I thank my family from the bottom of my heart for their encouragement and help.

January, 1998

The author

Original papers

Shoji Mano, Mokoto Hayashi and Mikio Nishimura (1997) Hydroxypyruvate reductase with a carboxy-terminal targeting signal to microbodies is expressed in *Arabidopsis*. *Plant Cell Physiol.* **38**: 449-455.

Shoji Mano, Katsushi Yamaguchi, Makoto Hayashi and Mikio Nishimura (1997) Stromal and thylakoid-bound ascorbate peroxidases are produced by alternative splicing in pumpkin. *FEBS Lett.* **413**: 21-26.

Shoji Mano, Makoto Hayashi and Mikio Nishimura (1998) Light regulates alternative splicing of hydroxypyruvate reductase in pumpkin. Submitted.

Plant Cell Physiol. 38(4): 449–455 (1997)

Hydroxypyruvate Reductase with a Carboxy-Terminal Targeting Signal to Microbodies is Expressed in *Arabidopsis*

Shoji Mano [1,2], Makoto Hayashi [1,2], Maki Kondo [1] and Mikio Nishimura [1,2,3]

[1] *Department of Cell Biology, National Institute for Basic Biology, Okazaki, 444 Japan*
[2] *Department of Molecular Biomechanics, School of Life Science, the Graduate University for Advanced Studies, Okazaki, 444 Japan*

Five *Arabidopsis* EST cDNA clones of hydroxypyruvate reductase (HPR), a photorespiratory enzyme in leaf peroxisomes, were sequenced. Deduced amino acid sequences revealed that HPR in *Arabidopsis* contained the carboxy-terminal targeting signal to microbodies. Nucleotide sequence analysis showed that the cDNA with the longest insert contained an open reading frame of 1,158 bp which encoded a polypeptide with 386 amino acids with a calculated molecular mass of 42,251 Da. A Southern blot analysis suggested that the *Arabidopsis* HPR gene, like that of the pumpkin HPR gene, exists as a single copy. Two kinds of pumpkin HPR mRNA might be produced from a single gene by alternative splicing, but the structure of the genomic DNA indicated that the *Arabidopsis* HPR gene did not undergo alternative splicing. We detected a polypeptide with a molecular mass of 42 kDa in green leaves of *Arabidopsis* using an HPR-specific antibody. Immunoelectron microscopy revealed that *Arabidopsis* HPR protein was exclusively localized in leaf peroxisomes in green leaves. These results indicate that HPR is expressed in a form with a carboxy-terminal targeting signal to microbodies and is localized in microbodies in *Arabidopsis*, suggesting that the differences in the gene structure and the regulation of gene expression of HPR are probably due to species-specific differences in plants.

Key words: Alternative splicing — *Arabidopsis thaliana* — Hydroxypyruvate reductase (EC 1.1.1.29) — Leaf peroxisome — Targeting signal.

NADH-hydroxypyruvate reductase (HPR; EC 1.1.1.29), which is one of the leaf-peroxisomal enzymes, catalyzes the reduction of hydroxypyruvate to glycerate. This enzyme is known to be localized in leaf peroxisomes, which are specialized microbodies found in photosynthetic tissues, and they play a role in the glycolate pathway of photorespiration in concert with enzymatic reactions in chloroplasts and mitochondria (Tolbert et al. 1968). HPR activity and transcripts are developmentally regulated and enhanced by light (Greenler et al. 1989) and similar results have been observed for other leaf-peroxisomal enzymes such as glycolate oxidase (Tsugeki et al. 1993). cDNAs for HPR have been cloned from pumpkin (Hayashi et al. 1996b), cucumber (Greenler et al. 1989) and methylotrophs (Chistoserdova and Lidstrom 1994, Yoshida et al. 1994). In plants, HPR functions in the photorespiratory glycolate pathway, whereas in methylotrophs, it acts as an assimilatory enzyme of one-carbon components, such as methanol, in the serine pathway.

Previous work in our laboratory has shown that two kinds of cDNA clones for HPR (HPR1 and HPR2) were screened from the cDNA library in pumpkin green cotyledons (Hayashi et al. 1996b). The nucleotide sequences of these clones are identical except for three single-nucleotide substitutions and for the region encoding the carboxy terminus. The deduced HPR1 protein contains the carboxy-terminal tripeptide of Ser-Lys-Leu, which is known as a targeting signal to microbodies (Gould et al. 1988, 1989) but the deduced HPR2 protein does not. These results suggested that HPR1 protein is transported into leaf peroxisomes and HPR2 protein remains in the cytosol. Moreover, analysis of the structure of an intron suggests that alternative splicing gives rise to two kinds of HPR mRNA. We have detected two polypeptides in homogenates from pumpkin leaves (see results), and have confirmed that one of them is localized in leaf peroxisomes and the other is localized in the cytosol of green leaves (Mano, Hayashi, Kondo and Nishimura. unpublished).

Alternative splicing is a well-known post-transcriptional regulatory mechanism in eukaryotic organisms. It has been reported that the synthesis of several enzymes are controlled by alternative splicing (Corti et al. 1994, Görlach et al. 1995, Hayashi et al. 1996b, Kopriva et al. 1995, Sugiyama et al. 1996, Tamaoki et al. 1995, Theill et al. 1992). Recent analysis from mammalian showed the presence of SR proteins, which are regarded as candidates for alternative splicing factor (Gontarek and Derse 1996, Screaton et al. 1995). Lopato et al. (1996) demonstrated that plants also possessed SR proteins. These SR proteins from carrot, tobacco and *Arabidopsis* were active in an alternative splicing assay in vitro. Moreover, the existence of exonic splicing enhancers, which are sequences to which SR proteins are able to bind, has been demonstrated (Gontarek and

The nucleotide sequence reported in this paper has been submitted to the DDBJ, EMBL and GenBank nucleotide sequence databases with the accession number D85339.
[3] To whom correspondence should be addressed.

Derse 1996).

In the case of peroxisomal enzymes, different forms of human carnitine acetyltransferase are produced by alternative splicing and are transported into distinct organelles (peroxisomes and mitochondria) (Corti et al. 1994). In higher plants, pumpkin HPR is the first example that microbody enzymes produced by alternative splicing are localized in different parts within cells. cDNA for cucumber HPR, which is another registered cDNA for HPR in plants, does not have this carboxy-terminal tripeptide. However, the genomic structure of cucumber suggested the possibility of alternative splicing (Hayashi et al. 1996b), suggesting that cucumber HPR with the microbody targeting signal exists, although the cDNA has not been cloned. Therefore, it is of interest to analyze the structure and the expression of HPR in another plant species.

In this paper, we report the characterization of *Arabidopsis* HPR for the purpose of investigating whether HPR is regulated by alternative splicing as it is pumpkin. Several EST clones homologous to pumpkin HPR have been already registered at the Arabidopsis Biological Resource Center (ABRC) at Ohio State University. We obtained these clones and determined the sequences to identify whether these have the targeting signal at their carboxy terminus. In addition, we also report here the results of a Southern blot analysis, the characterization of genomic DNA and the intracellular localization of the HPR protein in *Arabidopsis* green leaves. Overall, the analyses indicate that *Arabidopsis* HPR is a single-copy gene and is not regulated by alternative splicing, showing that only HPR with a carboxy-terminal targeting signal to microbodies is expressed and localized in microbodies in *Arabidopsis*.

Materials and Methods

Plant materials—Seeds of *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia were grown at 22°C under continuous light. Pumpkin (*Cucurbita* sp. cv. Kurokawa Amakuri Nankin) seeds were soaked overnight and germinated in moist rock fiber (66R; Nitto Bouseki, Chiba, Japan) at 25°C under continuous light.

Sequencing of Arabidopsis HPR EST clones—Five *Arabidopsis* HPR EST clones, whose stock numbers were 60A7T7, 135P15T7, 113J10T7, 183L19T7 and 133M20T7, were obtained from ABRC. These were derived from a λZip-Lox plasmid (GIBCO BRL, MD, U.S.A.). The nucleotide sequences encoding around the carboxy-terminal region of each clone and the full-length sequence of the longest cDNA insert, designated 113J10T7, were determined with an automatic DNA sequencer (model 373; Perkin Elmer/Applied Biosystems) according to the manufacturer's instructions. The nucleotide and the deduced amino acid sequences were analyzed with DNA analytical software (GeneWorks; IntelliGenetics, Mountain View, CA).

Cloning of genomic DNA for Arabidopsis HPR—An intron of the gene for *Arabidopsis* HPR was amplified by polymerase chain reaction (PCR) using total genomic DNA (5 ng) as a template. The genomic DNA was extracted from *Arabidopsis* leaves by the cetyltrimethylammonium bromide precipitation method (Murray and Thompson 1980). The reaction mixture contained 1 unit of Ampli Taq DNA polymerase (Perkin Elmer Japan, Chiba, Japan), an upstream primer (ATHPR1; 5′-GCATCGTCAACTCAAAGGCCTTA-3′), a downstream primer (ATHPR2; 5′-TACATAAACACAACTTGACACC-3′) and an appropriate buffer in a total volume of 50 μl. Each cycle was at 94°C for 45 s, 55°C for 45 s and 72°C for 45 s. The DNA fragment was subcloned into a T-vector prepared using pBluescript KS + as described in a previous report (Marchuk et al. 1990).

Southern blot analysis—Total DNA (1 μg) was digested with *Bgl*II and *Xba*I. The products were fractionated on a 0.8% agarose gel, transferred to a Zeta-Probe blotting membrane (BIO-RAD, CA, U.S.A.) by the capillary method and fixed by exposure to UV-light (Funa-UV-Linker, model FS-800; Funakoshi, Tokyo, Japan). The fragment corresponding to *Arabidopsis* HPR EST clone (113J10T7) was labeled with [^{32}P]dCTP (Amersham Japan) using a *Bca*Best labeling kit (Takara Shuzo, Kyoto, Japan). The membrane was hybridized in 50% formamide, 0.12 M sodium phosphate (pH 7.2), 0.25 M sodium chloride, 7% SDS and 1 mM EDTA (pH 8.0) with 1.0×10^6 cpm ml^{-1} of radiolabeled DNA probe for 18 h at 42°C. The membrane was washed at 42°C in 2× SSC plus 0.1% SDS for 15 min, in 0.2×SSC plus 0.1% SDS for 15 min, in 0.1×SSC plus 0.1% SDS for 15 min. The membrane was used to expose X-ray film.

Immunoblot analysis—The leaves of *Arabidopsis* plants and pumpkin cotyledons at the 21-old-day stage were homogenized with the extraction buffer (100 mM Tris-HCl, pH 6.8, 1 mM EDTA, pH 8.0, 0.1% Triton X-100 and 1 mM PMSF) and then the homogenates were centrifuged at 15,000×*g* for 25 min. The protein content of each extract was estimated by using a protein assay kit (Nippon Bio-Rad Laboratories, Tokyo, Japan) with bovine gamma albumin as a standard protein. Five or ten μg of total protein was separated by SDS-PAGE on a 7.5% polyacrylamide gel as described by Laemmli (1970) and transferred to a nylon membrane (Schleicher & Schuell, Dassel, Germany) in a semidry electroblotting system. Immunologic reactions were detected by monitoring the activity of horseradish peroxidase antibodies against rabbit IgG (ECL system; Amersham, Japan).

Immunoelectron microscopy—*Arabidopsis* leaves at the 21-day-old stage were fixed, dehydrated and embedded in LR white resin (London Resin, U.K.) as described previously (Nishimura et al. 1993). Ultra-thin sections were cut on a Reichert ultramicrotome (Leica, Heidelberg, Germany) with a diamond knife and mounted on uncoated nickel grids. The protein A-gold labeling procedure was essentially the same as that described by Nishimura et al. (1993). Ultra-thin sections were incubated at room temperature for 1 h with a solution of antiserum against HPR diluted 1 : 2,000 and then with a 50-fold diluted suspension of protein A-gold (Amersham Japan) at room temperature for 30 min. The sections were examined with a transmission electron microscope (1200EX; JOEL, Tokyo, Japan) at 80 kV.

Results and Discussion

Full-length sequence of Arabidopsis HPR cDNA—An EST bank at ABRC at Ohio State University was screened for HPR cDNA in *Arabidopsis* using the amino acid sequence of pumpkin HPR1 protein. Five cDNA clones showed high similarities to pumpkin HPR1. The stock numbers of these clones were 60A7T7 (accession No. T41584), 135P15T7 (accession No. T75991), 113J10T7 (accession No. T42460), 183L19T7 (accession No. H37101) and

133M20T7 (accession No. T45536). The complete nucleotide sequence of 113J10T7, which contained the longest insert, was determined. As shown in Figure 1, this cDNA consists of 1,428 bp and contains a 1,158-bp open reading frame that encodes a polypeptide with 386 amino acids with a total molecular mass of 42,251 Da. The deduced sequence beginning with the first ATG at nucleotide 81 showed high similarities to HPRs from other sources such as pumpkin (Hayashi et al. 1996b), cucumber (Greenler et al. 1989) and methylotrophs (Chistoserdova and Lidstrom 1994, Yoshida et al. 1994). The identities with amino acid sequences of HPR for other plants are 88% for pumpkin HPR1, 87% for pumpkin HPR2 and 87% for cucumber. The carboxy-terminal sequence was Ser-Lys-Leu, which is known to be one of the targeting signals to microbodies (Gould et al. 1988, 1989).

Nucleotide sequences around the carboxy terminus of Arabidopsis EST clones—The carboxy-terminal targeting signals to microbodies are characterized well in glyoxysomal- and peroxisomal-enzymes (Gould et al. 1988, 1989,

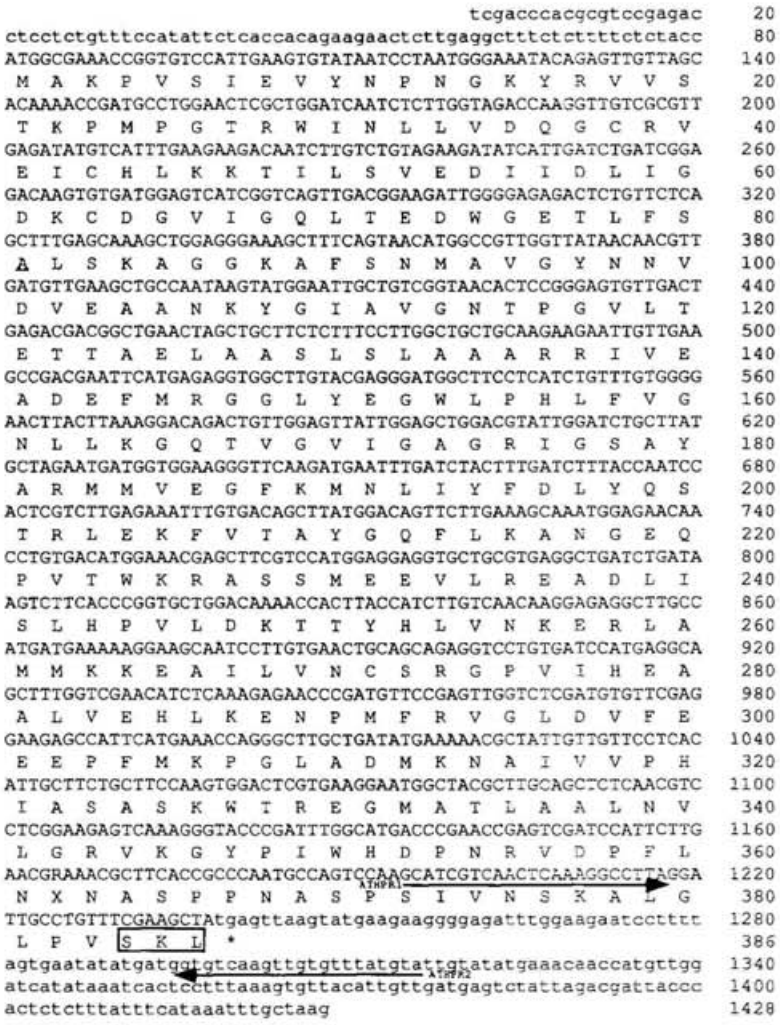


Fig. 1 Nucleotide and deduced amino acid sequences of *Arabidopsis* HPR cDNA. The complete nucleotide sequence of 113J10T7 was determined. The nucleotide sequence of the putative coding region is shown in uppercase letters and the 5′- and 3′-noncoding regions are shown in lowercase letters. The deduced amino acid sequence is presented in the single-letter code under the nucleotide sequence, starting at the first in-frame methionine residue and ending at the first stop codon, indicated by an asterisk. The three carboxy-terminal amino acids that are known as a microbody-targeting signal are boxed. Arrows indicate primers, ATHPR1 and ATHPR2, for PCR, respectively.

Hayashi et al. 1996a, Mano et al. 1996, Mori et al. 1991, Olsen et al. 1993, Tsugeki et al. 1993, Volkita 1991). But another HPR protein in pumpkin (HPR2) does not have this signal. We determined the nucleotide sequences encoding the region around the carboxy terminus of other clones in order to identify whether or not these sequences contained the targeting signal to microbodies. As shown in Figure 2, the nucleotide sequences of all cDNAs were identical, although the lengths and positions of the poly-(A) tail were different. All deduced amino acid sequences contained the Ser-Lys-Leu sequence at the carboxy terminus and none of the HPRs were found to lack the targeting signal, as occurs in pumpkin HPR2 cDNA.

Genomic sequence and putative amino acid sequence of Arabidopsis HPR—We could not exclude the possibility of the presence of another type of cDNA in *Arabidopsis* such as pumpkin HPR2 cDNA and cucumber HPR cDNA (Greenler et al. 1989), which lacked the Ser-Lys-Leu sequence at their carboxy terminus, because it might not be registered in the EST bank. We have reported that HPR without the targeting signal may be produced from one gene by alternative splicing since the genomic structure contains two pairs of GT-AG doublets (Hayashi et al. 1996b), so two kinds of mRNA are produced depending on the way in which the exons are spliced. It is assumed that the longer nucleotide sequence flanked by GT-AG is removed as it is in the case of HPR1. HPR2 is produced when the shorter intron, which does not contain the first 17 bp with a stop codon, is eliminated. There is a similar structure around intron *XII* of cucumber genomic DNA (Hayashi et al.

```
                    L  G  L  P  V  S  K  L  *
60A7T7          CCTTAGGATTGCCTGTTTCGAAGCTAtgagttaagtatgaagaaggggag
135P15T7        CCTTAGGATTGCCTGTTTCGAAGCTAtgagttaagtatgaagaaggggag
113J10T7        CCTXAGGATTGCCXGTTTCGAAGCTAtgagttaagtatgaagaaggggag
183L19T7        CCTXAGGATTGCCXGTTTCGAAGCTAtgagttaagtatgaagaaggggag
113M20T7        TTTCAGGATTGCCTGTTTCGAAGCTAtgagttaagtatgaagaaggggag
                  * ********* ************************************

60A7T7          atttggaagaatccttttagtgaatatatgatggtgtcaagttgtgttta
135P15T7        atttggaagaatccttttagtgaatatatgatggtgtcaagttgtgttta
113J10T7        atttggaagaatccttttagtgaatatatgatggtgtcaagttgtgttta
183L19T7        atttggaagaatccttttagtgaatatatgatggtgtcaagttgtgttta
113M20T7        atttggaagaatccttttagtgaatatatgatggtgtcaagttgtgttta
                ************************************************

60A7T7          tgtattgtatatgaaacaaccatgttggatcatataaatcactcctttaa
135P15T7        tgtattgtatatgaaacaaccatgttggatcatataaatcactcctttaa
113J10T7        tgtattgtatatgaaacaaccatgttggatcatataaatcactcctttaa
183L19T7        tgtattgtatatgaaacaaccatgttggatcatataaatcactcctttaa
113M20T7        tgtattgtatatgaaacaaccatgttggatcatataaatcactcctttaa
                ************************************************

60A7T7          agtgttacattgttgatgagtctattagacgattacccactctctttatt
135P15T7        agtgttacattaaaaaaaaaaaaaa
113J10T7        agtgttacattgttgatgagtctattagacgattacccactctctttatt
183L19T7        agtgttacattgttgatgagtctattagacgattacccactctctttatt
113M20T7        agtgttacattgttgatgagtctattagacgattacccactctctttatt
                ***********

60A7T7          tcataaatttgctaagaatacaatttctaagtcaaaaaaaaaaaaaaaaa
113J10T7        tcataaatttgctaagaatacaaaaaaaaaaaaaaaaaaaaaa
183L19T7        tcataaatttgctaagaatacaaaaaaaaaaaaaaaaaaaaaa
113M20T7        tcataaatttgctaagaatacaatttctaagtcatcaagaaaaaaaaaaa


60A7T7          aaa
113M20T7        aaaaaaaaaaaa
```

Fig. 2 Alignment of nucleotide sequences encoding the carboxy-terminal amino acids of *Arabidopsis* EST clones. All clones were obtained from ABRC. The nucleotide sequence of the coding region is shown in uppercase letters and the 3′-noncoding region is shown in lowercase letters. The deduced amino acid sequence around the carboxy terminus is presented in the single-letter code over the nucleotide sequence. Identical nucleotides are indicated by an asterisk under the sequence. The stock number of each clone is shown on the left.

1996b). Therefore, it is important to compare the genomic structure of *Arabidopsis* HPR with those of pumpkin and cucumber HPRs. To characterize the structure of the *Arabidopsis* HPR gene, we amplified *Arabidopsis* genomic DNA by PCR. A unique fragment of about 300 bp in length was amplified, subcloned into pBS vector and then subjected to sequence analysis. The result showed a 188-bp intron located at the same position as it is in the HPR genes of pumpkin and cucumber (Fig. 3). An exon-intron junction followed the GT-AG rule and showed good agreement with the donor and acceptor consensus sequence derived for plant gene splice sites. We were unable to find two pairs of GT-AG doublets within this intron. We determined the nucleotide sequences of six independently isolated products of PCR amplified from the genomic DNA. All these DNAs had the same nucleotide sequence as shown in Figure 3. These data suggest that the gene for *Arabidopsis* HPR contains only one GT-AG doublet and that alternative splicing might not occur.

Arabidopsis HPR is encoded by a single copy gene— Since *Arabidopsis* HPR does not seem to be produced by alternative splicing, does another HPR gene exist in *Arabidopsis*? Givan and Kleczkowski (1992) detected the activities and polypeptides of HPR in leaf peroxisomes and the cytosol of barley leaves. Judging from the difference in subunit molecular masses between HPR1 and HPR2 in barley, they seem to be derived from different genes, whereas Greenler et al. (1989) reported that a single gene for HPR is present per haploid genome in cucumber, and we confirmed that pumpkin HPR was also a single-copy gene (Mano, Hayashi, Kondo and Nishimura, unpublished). Therefore, we carried out a Southern blot analysis. DNA from leaves of *Arabidopsis* was isolated, digested with *Bgl*II and *Xba*I, none of which cuts *Arabidopsis* cDNA (113J10T7), and subjected to Southern blot hybridization using *Arabidopsis* HPR cDNA as a probe. As shown in Figure 4, HPR cDNA hybridized with a single fragment. In addition, only one kind of genomic sequence was obtained when we amplified the genomic DNA (Fig. 3). These findings indicate that HPR in *Arabidopsis* exists as a single-copy gene, as it does

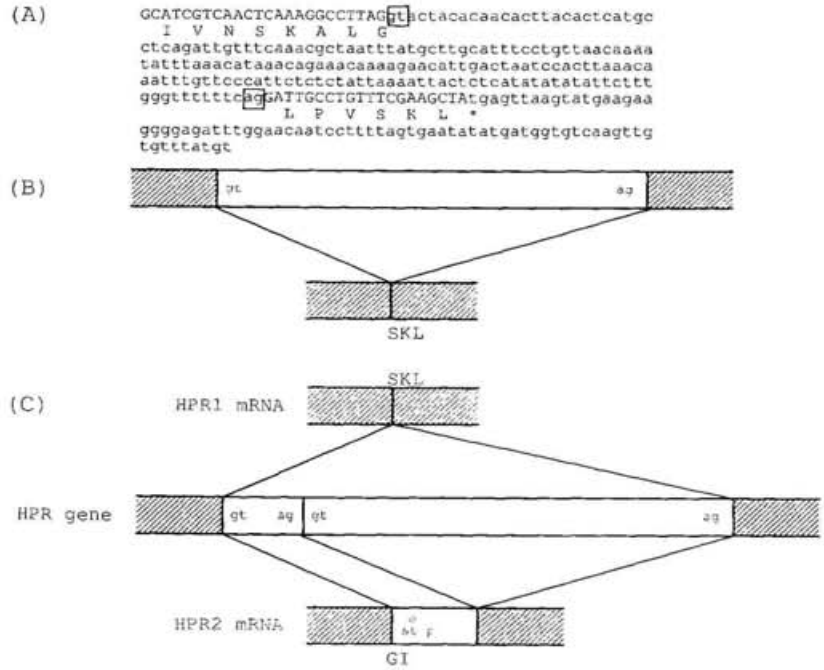


Fig. 3 Structural relationships around the carboxy termini of HPR from *Arabidopsis* and pumpkin. (A) The nucleotide sequence of the intron in this region of *Arabidopsis* HPR gene is shown. The nucleotide sequence of the exon is shown in uppercase letters and the intron and 3′-noncoding region are shown in lowercase letters. The deduced amino acid sequence is presented in the single-letter code under the nucleotide sequence. Asterisk indicates the stop codon and boxes represent the consensus sequences found in the beginning (GT) and the end (AG) of an intron. (B), (C) Schematic representation between the proteins deduced from *Arabidopsis* mRNA (B) and pumpkin mRNAs (C) and each gene. The hatched boxes correspond to exons and the open boxes correspond to introns. The consensus sequences corresponding to the splice donor and acceptor sites and the carboxy-terminal amino acids are shown. Asterisk shows the stop codon.

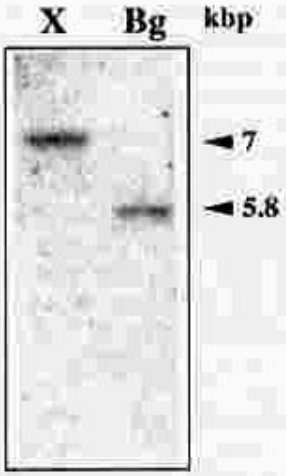


Fig. 4 Southern blot analysis of *Arabidopsis* genomic DNA. Genomic DNA (1 μg each) was digested with the restriction endonucleases indicated, separated on a 0.8% agarose gel, transferred to a Zeta-Probe blotting membrane (BIO-RAD) and probed with a [^{32}P]-labeled *Arabidopsis* HPR cDNA fragment. The size of DNA fragments are shown in kbp on the right. X, *Xba*I; Bg, *Bgl*II.

in pumpkin and cucumber.

Antibodies against HPR cross-react with a 42 kDa polypeptide—Immunoblotting was carried out using antibodies against spinach HPR. Total proteins prepared from *Arabidopsis* and pumpkin, as a reference, were subjected to SDS-PAGE. A unique polypeptide with a molecular mass of 42 kDa was recognized by the antibodies against spinach HPR in the total *Arabidopsis* extract (Fig. 5, lane 1 and 2). This is in good agreement with the molecular mass calculated from the deduced amino acids. In the case of total protein from pumpkin, two polypeptides were detected (Fig. 5, lane 3). We previously reported the presence of two kinds of HPR cDNA in green pumpkin cotyledons, which were designated HPR1 and HPR2 (Hayashi et al. 1996b). The molecular masses of the HPR1 and HPR2 proteins are calculated to be 42,305 Da and 41,709 Da from their deduced amino acids, respectively. This unique band in *Arabidopsis* did not split into two bands under the same electrophoretic condition, even though the volume of total protein was reduced (Fig. 5, lane 1). These data indicated that HPR in *Arabidopsis* is only expressed as a 42 kDa polypeptide. Based on these data, we postulate that *Arabidopsis* has one kind of HPR enzyme (from a single gene) and pumpkin has two kinds of HPR enzyme produced by alternative splicing. These results indicate that the structure of the HPR gene varies with plant species and that the regulation of expression of the HPR gene may be different.

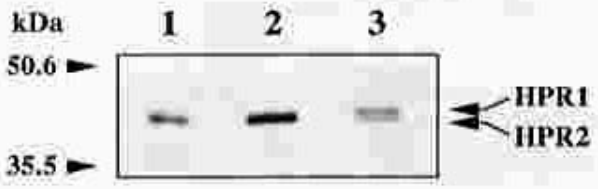


Fig. 5 Detection of HPR polypeptides in *Arabidopsis*. Total protein prepared from the leaves of *Arabidopsis* plants and pumpkin cotyledons was subjected to SDS-PAGE, which was followed by immunodetection with antibodies against HPR. Lane 1 and 2, 5 μg and 10 μg of total protein of *Arabidopsis* respectively; lane 3, 10 μg of total protein of pumpkin cotyledons. Molecular markers are indicated in kDa on the left. Arrows on the right represent the HPR1 and HPR2 polypeptides of pumpkin.

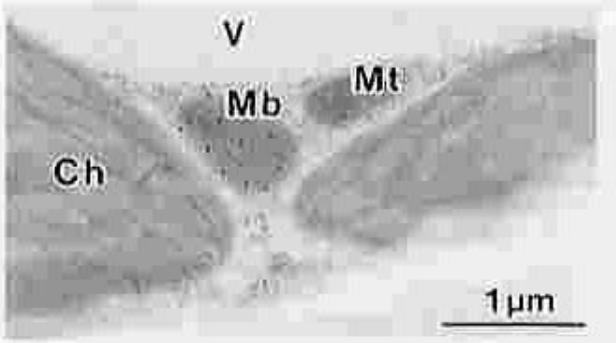


Fig. 6 Immunocytochemical localization of HPR in *Arabidopsis* and pumpkin. Immunogold labeling of ultra-thin sections of *Arabidopsis* leaves was carried out using antibodies against HPR. Mb, microbody; Mt, mitochondrion; Ch, chloroplast; V, vacuole. Bar indicates 1 μm.

Arabidopsis HPR is exclusively localized in leaf peroxisomes—The localization of HPR was investigated using immunoelectron microscopy. Gold particles for HPR were exclusively detected in leaf peroxisomes but not in other organelles such as mitochondria, chloroplasts and vacuoles (Fig. 6). This electron micrograph clearly shows that HPR is transported into leaf peroxisomes in *Arabidopsis*. This is in agreement with the result that *Arabidopsis* HPR is detected as one kind of polypeptide and that the expression of HPR is not regulated by alternative splicing. Further analysis will be required to understand the detailed mechanism of alternative splicing in higher plants.

We are most grateful to Dr. N.-H. Chua (Rockefeller University) for the antiserum against hydroxypyruvate reductase from spinach. This work was supported by a grant for "Research for the Future" Program (JSPS-RFTF 96L00407) from the Japan Society for the Promotion of Science and Grants-in-Aid for Scientific Research (No. 07456053, 04273101 and 04273102) from the Ministry of Education, Science and Culture, Japan, by a grant from the Nissan Science Foundation (Tokyo, Japan), by the NIBB program for Molecular Mechanisms of Stress Response and by a grant from the DAIKO Foundation (Nagoya, Japan).

References

Chistoserdova, L.V. and Lidstrom, M.E. (1994) Genetics of the serine cycle in *Methylobacterium extorquens* AM1: identification of *sga*A and *mtd*A and sequences of *sga*A, *hpr*A and *mtd*A. *J. Bacteriol.* 176: 1957–1968.

Corti, O., Didonato, S. and Finocchiaro, G. (1994) Divergent sequences in the 5′ region of cDNA suggest alternative splicing as a mechanism for the generation of carnitine acetyltransferases with different subcellular localizations. *Biochem. J.* 303: 37–41.

Givan, C.V. and Kleczkowski, L.A. (1992) The enzymic reduction of glyoxylate and hydroxypyruvate in leaves of higher plants. *Plant Physiol.* 100: 552–556.

Gontarek, R.R. and Derse, D. (1996) Interactions among SR proteins, an exonic splicing enhancer, and a Lentivirus Rev protein regulate alternative splicing. *Mol. Cell. Biol.* 16: 2325–2331.

Görlach, J., Raesecke, H.-R., Abel, G., Wehrli, R., Amrhein, N. and Schmid, J. (1995) Organ-specific differences in the ratio of alternatively spliced chorismate synthase (*LeCS2*) transcripts in tomato. *Plant J.* 8: 451–456.

Gould, S.J., Keller, G.-A., Hosken, N., Wilkinson, J. and Subramani, S. (1989) A conserved tripeptide sorts proteins to peroxisomes. *J. Cell Biol.* 108: 1657–1664.

Gould, S.J., Keller, G.-A. and Subramani, S. (1988) Identification of peroxisomal targeting signals located at the carboxy terminus of four peroxisomal proteins. *J. Cell Biol.* 107: 897–905.

Greenler, J.M., Sloan, J.S., Schwartz, B.W. and Becker, W.M. (1989) Isolation, characterization and sequence analysis of a full-length cDNA clone encoding NADH-dependent hydroxypyruvate reductase from cucumber. *Plant Mol. Biol.* 13: 139–150.

Hayashi, M., Aoki, M., Kato, A., Kondo, M. and Nishimura, M. (1996a) Transport of chimeric proteins that contain a carboxy-terminal targeting signal into plant microbodies. *Plant J.* 10: 225–234.

Hayashi, M., Tsugeki, R., Kondo, M., Mori, H. and Nishimura, M. (1996b) Pumpkin hydroxypyruvate reductases with and without a putative C-terminal signal for targeting to microbodies may be produced by alternative splicing. *Plant Mol. Biol.* 30: 183–189.

Kopriva, S., Cossu, R. and Bauwe, H. (1995) Alternative splicing results in two different transcripts for H-protein of the glycine cleavage system in the C_4 species *Flaveria trinervia*. *Plant J.* 8: 435–441.

Laemmli, U.K. (1970) Cleavage of structural proteins during assembly of head of bacteriophage T4. *Nature* 227: 680–685.

Lopato, S., Mayeda, A., Krainer, A.R. and Barta, A. (1996) Pre-mRNA splicing in plants: characterization of Ser/Arg splicing factors. *Proc. Natl. Acad. Sci. USA* 93: 3074–9079.

Mano, S., Hayashi, M., Kondo, M. and Nishimura, M. (1996) cDNA cloning and expression of a gene for isocitrate lyase in pumpkin cotyledons. *Plant Cell Physiol.* 37: 941–948.

Marchuk, D., Drumm, M., Saulino, A. and Collins, F.S. (1990) Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. *Nucl. Acids Res.* 19: 1154–1154.

Mori, H., Takeda-Yoshikawa, Y., Hara-Nishimura, I. and Nishimura, M. (1991) Pumpkin malate synthase. Cloning and sequence of the cDNA and Northern blot analysis. *Eur. J. Biochem.* 197: 331–336.

Murray, M.G. and Thompson, W.F. (1980) Rapid isolation of higher molecular weight plant DNA. *Nucl. Acids Res.* 8: 4321–4325.

Nishimura, M., Takeuchi, Y., Bellis, L.D. and Hara-Nishimura, I. (1993) Leaf peroxisomes are directly transformed to glyoxysomes during senescence of pumpkin cotyledons. *Protoplasma* 175: 131–137.

Olsen, L.J., Ettinger, W.F., Damsz, B., Matsudaira, K., Webb, M.A. and Harada, J.J. (1993) Targeting of glyoxysomal proteins to peroxisomes in leaves and roots of a higher plant. *Plant Cell* 5: 941–952.

Screaton, G.R., Cáceres, J.F., Mayeda, A., Bell, M.V., Plebanski, M., Jackson, D.G., Bell, J.I. and Krainer, A.R. (1995) Identification and characterization of three members of the human SR family of pre-mRNA splicing factors. *EMBO J.* 14: 4336–4349.

Sugiyama, T., Nishio, Y., Kishimoto, T. and Akira, S. (1996) Identification of alternative splicing form of Stat2. *FEBS Lett.* 381: 191–194.

Tamaoki, M., Tsugawa, H., Minami, E., Kayano, T., Yamamoto, N., Kano-Murakami, Y. and Matsuoka, M. (1995) Alternative RNA products from a rice homeobox gene. *Plant J.* 7: 927–938.

Theill, L.E., Hattori, K., Lazzaro, D., Castrillo, J.-L. and Karin, M. (1992) Differential splicing of the *GHF1* primary transcript gives rise to two functionally distinct homeodomain proteins. *EMBO J.* 11: 2261–2269.

Tolbert, N.E., Oeser, A., Kisaki, T., Hagemen, R.H. and Yamasaki, R.K. (1968) Peroxisomes from spinach leaves containing enzymes related to glycolate metabolism. *J. Biol. Chem.* 243: 5179–5184.

Tsugeki, R., Hara-Nishimura, I., Mori, H. and Nishimura, M. (1993) Cloning and sequencing of cDNA for glycolate oxidase from pumpkin cotyledons and Northern blot analysis. *Plant Cell Physiol.* 34: 51–57.

Volkita, M. (1991) The carboxy-terminal end of glycolate oxidase directs a foreign protein into tobacco leaf peroxisomes. *Plant J.* 1: 361–366.

Yoshida, T., Yamaguchi, K., Hagishita, T., Mitsunaga, T., Miyata, A., Tanabe, T., Toh, H., Ohshiro, T., Shimao, M. and Izumi, Y. (1994) Cloning and expression of the gene for hydroxypyruvate reductase (D-glycerate dehydrogenase) from an obligate methylotroph *Hyphomicrobium methylovorum* GM2. *Eur. J. Biochem.* 223: 727–732.

(Received November 11, 1996; Accepted February 1, 1997)

Stromal and thylakoid-bound ascorbate peroxidases are produced by alternative splicing in pumpkin

Shoji Mano[a,b], Katsushi Yamaguchi[a], Makoto Hayashi[a,b], Mikio Nishimura[a,b,*]

[a]*Department of Cell Biology, National Institute for Basic Biology, Okazaki 444, Japan*
[b]*Department of Molecular Biomechanics, School of Life Science, the Graduate University for Advanced Studies, Okazaki 444, Japan*

Received 13 May 1997

Abstract A cDNA encoding stromal ascorbate peroxidase (sAPX) was isolated using poly(A)$^+$ RNA of pumpkin cotyledons by RT-PCR. The cDNA encodes a polypeptide with 372 amino acids and shares complete sequence identity with pumpkin thylakoid-bound ascorbate peroxidase (tAPX), except for the deletion of a putative membrane spanning region located in the carboxy domain of tAPX. Southern blot hybridization and analysis of intron structure indicated that mRNAs for sAPX and tAPX, whose suborganellar localizations in chloroplasts are different, are produced by alternative splicing. Immunoblot analysis showed that the accumulation of sAPX and tAPX was differently regulated during germination and subsequent greening of pumpkin cotyledons.

Key words: Alternative splicing; Chloroplast; Pumpkin (*Cucurbita* sp. cv. Kurokawa Amakuri Nankin); Stromal ascorbate peroxidase (EC 1.11.1.11); Thylakoid-bound ascorbate peroxidase

1. Introduction

Ascorbate peroxidase (APX), which uses ascorbate as the electron donor, has a role in the scavenging of H_2O_2. In higher plants, four isoenzymes of APX with different subcellular localizations have been reported, namely, microbody APX (mbAPX) [1,2], cytosolic APX (cAPX) [3,4], stromal APX (sAPX) [5,6] and thylakoid-bound APX (tAPX) [6,7]. cAPX is localized in the cytosol of both photosynthetic and non-photosynthetic tissues. The function is still obscure, although it has been reported that mRNA for cAPX was induced by environmental stimuli such as drought and heat stress [8,9]. mbAPX is localized on glyoxysomal and leaf-peroxisomal membranes and plays a role in the reduction of H_2O_2 which leaks from glyoxysomes and leaf peroxisomes [1]. Chloroplastic APXs (sAPX and tAPX) scavenge the H_2O_2 within chloroplasts. Chloroplastic APXs have been purified and determined their partial amino acid sequences [5,7,10]. Recently, cDNA for tAPX from pumpkin has been cloned and characterized in our laboratory [11] and Ishikawa et al. [6] also isolated cDNAs for tAPX and sAPX from spinach leaves, showing that amino acid sequences of tAPX and sAPX were identical except for the deletion of the C-terminal domain of tAPX, although both APXs had a putative transit peptide into chloroplasts.

In this paper, we report the characterization of pumpkin sAPX for the purpose of investigating whether sAPX and tAPX are produced by alternative splicing. We isolated cDNA for sAPX from greening pumpkin cotyledons. A Southern blot analysis and the characterization of genomic DNA revealed that the pumpkin gene encoding sAPX and tAPX is a single copy and both APXs were produced by alternative splicing. We also report here the developmental changes in the level of sAPX and tAPX proteins during germination and subsequent greening of pumpkin cotyledons.

2. Materials and methods

2.1. Plant materials

Pumpkin (*Cucurbita* sp. cv. Kurokawa Amakuri Nankin) seeds were soaked overnight and germinated in moist rock fiber (66R; Nitto Bouseki, Chiba, Japan) at 25°C in darkness. Some of these seedlings were transferred to continuous illumination at the 5-day stage.

2.2. Sequencing of cDNA for pumpkin sAPX

First-strand cDNA was generated using a Ready-To-Go T-primed first-strand kit (Pharmacia Biotech, Tokyo, Japan) from poly(A)$^+$ RNA template prepared from 5-day dark- and 4-day light-grown cotyledons using Oligotex-dT 30 <Super> (Roche Japan, Tokyo, Japan). PCR was used to isolate full-length cDNA for pumpkin sAPX. The reaction mixture contained 1 U of Ampli Taq DNA polymerase (Perkin Elmer Japan, Chiba, Japan), an upstream primer (ASA-S1; 5′-ATCGCGTTCATTGCCAGTTG-3′), a downstream primer (SAPX-II; 5′-GACACGGTCACAACAAAACATG-3′) which was synthesized corresponding to the genomic sequence obtained previously (Fig. 2) and an appropriate buffer in a total volume of 50 μl. Each cycle was at 94°C for 45 s, 55°C for 45 s and 72°C for 45 s. The DNA fragment was subcloned into a T-vector prepared using pBluescript KS+ as described previously [12]. The nucleotide sequence was determined with an automatic DNA sequencer (model 377; Perkin Elmer/Applied Biosystems) according to the manufacturer's instructions. The nucleotide and the deduced amino acid sequences were analyzed with DNA analytical software (GeneWorks; IntelliGenetics, Mountain View, CA).

2.3. Cloning of genomic DNA for pumpkin sAPX

An intron of the gene for pumpkin sAPX (tAPX) was amplified by PCR using total genomic DNA (5 ng) as a template. The genomic DNA was extracted from pumpkin leaves by the cetyltrimethylammonium bromide precipitation method [13]. The reaction mixture contained 1 U of Ampli Taq DNA polymerase (Perkin Elmer Japan, Chiba, Japan), an upstream primer (pumpchl A; 5′-GGTATTGTGATTGATGATGC-3′), a downstream primer (3′-non; 5′-GGATCGTGAAATCAGAAGACA-3′) and an appropriate buffer in a total volume of 50 μl. Each cycle was at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. Cloning and sequencing of the DNA were carried out as described previously (see Section 2.2).

*Correspondence author. Department of Cell Biology, National Institute for Basic Biology, Myodaiji, Okazaki 444, Japan. Fax: 81-564-55-7505.

Abbreviations: APX, ascorbate peroxidase; sAPX, stromal APX; tAPX, thylakoid-bound APX; mbAPX, microbody APX; cAPX, cytosolic APX; HPR, hydroxypyruvate reductase; RT-PCR, reverse transcription-polymerase chain reaction

The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with the accession number D88420.

PII S0014-5793(97)00862-4

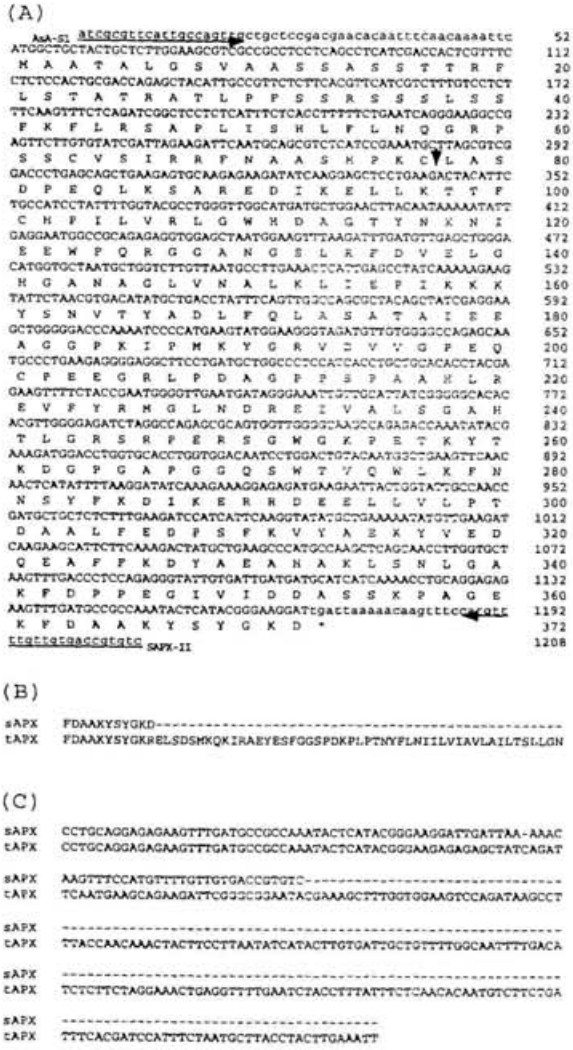


Fig. 1. Nucleotide and deduced amino acid sequences of pumpkin sAPX cDNA. A: The nucleotide sequence of the putative coding region is shown in uppercase letters and the 5′- and 3′-noncoding regions are shown in lowercase letters. The deduced amino acid sequence is presented in the single-letter code under the nucleotide sequence, starting at the first in-frame methionine residue and ending at the first stop codon, indicated by an asterisk. Arrowhead indicates the putative site of cleavage of the transit peptide, as estimated from the N-terminal sequence of tea sAPX and spinach tAPX. Primers for PCR (ASA-S1 and SAPX-II) are underlined with an arrowhead indicating the polarity. B: Comparison of the deduced amino acid sequences of the carboxy termini of sAPX and tAPX [11]. C: Comparison of the nucleotide sequences encoding the carboxy termini of sAPX and tAPX [11].

2.4. Southern blot analysis

Total DNA (10 μg) was digested with *Mlu*I, *Sal*I, *Xho*I and *Bam*HI. The products were fractionated on a 0.8% agarose gel, transferred to a Zeta-Probe blotting membrane (Bio-Rad, CA) by the capillary method and fixed by exposure to UV-light (Funa-UV-Linker, model FS-800; Funakoshi, Tokyo, Japan). The fragment corresponding to pumpkin sAPX was labeled with [^{32}P]dCTP (Amersham, Japan) using a Megaprime DNA labeling system (Amersham, Japan). The membrane was hybridized in 50% formamide, 0.12 M sodium phosphate (pH 7.2), 0.25 M sodium chloride, 7% SDS and 1 mM EDTA (pH 8.0) with 1.0×10^6 cpm·ml^{-1} of radiolabeled DNA probe for 18 h at 42°C. The membrane was washed at 42°C in 2×SSC plus 0.1% SDS for 15 min, in 0.2×SSC plus 0.1% SDS for 15 min, in 0.1×SSC plus 0.1% SDS for 15 min. The membrane was used to expose to the imaging plate of a Bio-Imaging analyzer (FUJIX BAS2000; FUJI Photo Film, Tokyo) with an exposure time of 18 h.

2.5. RT-PCR analysis

First-strand cDNA was synthesized as described previously (see Section 2.2) using poly(A)$^+$ RNA prepared from 5-day dark- and 2-day light-grown cotyledons. PCR was performed as described previously (see Section 2.2) except for EX Taq DNA polymerase (Takara Shuzo, Kyoto, Japan) and specific primers; the 5′-common sense primer of sAPX and tAPX (ASA3; 5′-CCTGGACTGTACAATGG-3′) and the 3′-specific antisense primers for tAPX (3′-non; see Section 2.3) and sAPX (SAPX-I; 5′-AGACGCGGACATCACAGATGTC-3′). The condition were 25 cycles of 45s at 94°C, 45s at 52°C and 45s at 72°C.

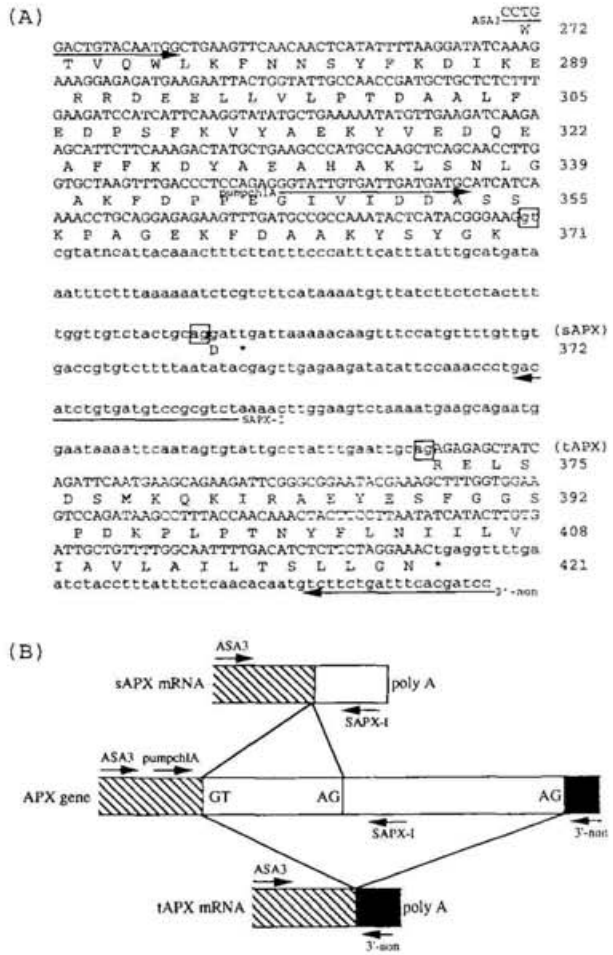


Fig. 2. A: The nucleotide sequence around the carboxy terminal of the pumpkin chloroplastic *APX* gene is shown. The nucleotide sequence of the exon is shown in uppercase letters and the intron and 3′-noncoding region are shown in lowercase letters. The deduced amino acid sequence is presented in the single-letter code under the nucleotide sequence with numberings on the right. Asterisks indicate the stop codons and boxes represent the consensus sequences found in the beginning (GT) and the end (AG) of an intron. PCR primers. ASA3, pumpchl A, SAPX-I and 3′-non, are underlined with an arrowhead indicating the polarity. B: Schematic representation of mRNAs and the gene. The hatched and filled boxes correspond to exons and the open boxes correspond to introns. Primers for PCR and the consensus sequences corresponding to the splice donor and acceptor sites are shown.

2.6. Immunoblot analysis

Pumpkin cotyledons grown under various conditions were homogenized with SDS-loading buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 0.2 mM NaCl and 10% SDS) and then the homogenates were centrifuged at 12 000× rpm for 10 min. Supernatants were subjected to SDS-PAGE on a 10% polyacrylamide gel as described by Laemmli [14] and transferred to a nylon membrane (Schleicher and Schuell, Dassel, Germany) in a semidry electroblotting system. Antibodies against pumpkin mbAPX prepared as described previously [1] were used and the polypeptides on the membrane were visualized with 1:5000 dilution of horseradish peroxidase antibodies against rabbit IgG (ECL System; Amersham, Japan).

3. Results and discussion

sAPX was cross-reacted with antibodies against the carboxy terminal 82-residue polypeptide of tAPX but cAPX and mbAPX were not [11]. The immunoreactivity suggests that sAPX contains homologous sequences to tAPX. Furthermore, we had determined the genomic sequence around the carboxy terminal region of tAPX (Fig. 2A). The genomic sequence showed the possibility that tAPX and sAPX are produced by alternative splicing. Therefore, RT-PCR was performed to amplify the coding region of pumpkin sAPX using primers of ASA-S1 and SAPX-II (Fig. 1). The 1.2-kb product was cloned into pBS vector and sequenced. The complete nucleotide and deduced amino acid sequences are shown in Fig. 1A. The cDNA consists of 1208 bp and contains a 1116-bp open reading frame that encodes a polypeptide with 372 amino acids with a total molecular mass of 40 661 Da. In the case of cleavage of the transit peptide on the C-terminal side of Cys-77, the calculated total molecular mass is 32 594 Da. This is in good agreement with the molecular mass of polypeptides recognized by antibodies against pumpkin mbAPX [11]. The nucleotide and deduced amino acid sequences of sAPX have been reported for spinach [6] and *Arabidopsis* (unpublished, accession no. X98925). The pumpkin sAPX was 76% identical with spinach sAPX. The sequence of the cDNA showed com-

plete identity with that of pumpkin tAPX cDNA [11] except for the carboxy terminal region, which contains a putative thylakoid-spanning domain (Fig. 1B,C). sAPX contained the transit peptide to chloroplasts but the lack of the thylakoid-spanning domain may make it remain in the stroma. The complete identity except for the carboxy terminal region between sAPX cDNA and tAPX cDNA suggests that alternative splicing causes a single gene to give rise to two mRNAs whose translated products are localized in different parts within cells. In higher plants, it has been reported that the synthesis of several enzymes are controlled by alternative splicing [6,15–20]. To investigate whether sAPX and tAPX in pumpkin are also produced by alternative splicing, we amplified pumpkin genomic DNA around the carboxy terminal region by PCR. A unique fragment of about 550 bp in length was amplified, subcloned into pBS vector and sequenced. The sequence showed the presence of a 291-bp intron that contains one donor site and two acceptor sites (Fig. 2A), suggesting that the synthesis of sAPX mRNA or tAPX mRNA depends on which acceptor site is used for the splicing (Fig. 2B), as occurs with the rat A3 adenosine receptor mRNA [21]. The nucleotide sequences of independently isolated products of PCR amplified from the genomic DNA had the same nucleotide sequence as shown in Fig. 2A.

To identify whether another *sAPX* gene exists, DNA from leaves of pumpkin was isolated, digested with *Mlu*I, *Sal*I and *Xho*I (none of which cuts pumpkin sAPX cDNA) and with *Bam*HI (which cuts the cDNA at one site) and then subjected to Southern blot hybridization using pumpkin sAPX cDNA as a probe. As shown in Fig. 3, the sAPX probe hybridized to a single band in the case of the digestion with *Mlu*I, *Sal*I and

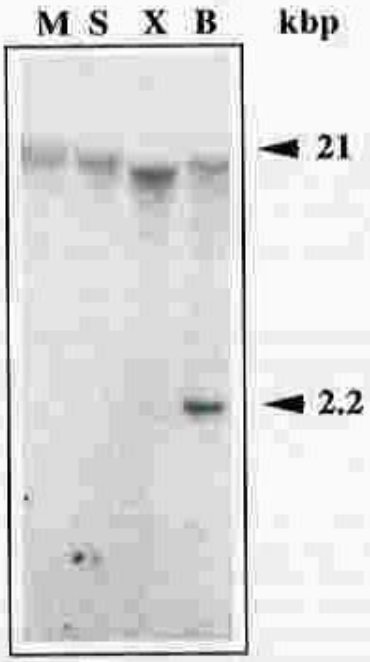


Fig. 3. Southern blot analysis of pumpkin genomic DNA. Genomic DNA (10 μg each) was digested with the restriction endonucleases indicated, separated on a 0.8% agarose gel, transferred to a Zeta-Probe blotting membrane (Bio-Rad) and probed with a ^{32}P-labeled pumpkin sAPX cDNA fragment. The size of DNA fragments are shown in bp on the right. M, *Mlu*I; S, *Sal*I; X, *Xho*I; B, *Bam*HI.

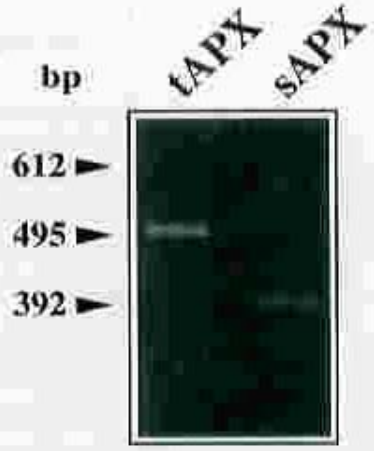


Fig. 4. PCR amplification of truncated sAPX- and tAPX-specific mRNA. RT-PCR was carried out using mRNAs prepared from 5-day dark- and 2-day light-grown cotyledons. The products were analyzed by electrophoresis on a 1% agarose gel and stained with ethidium bromide.

*Xho*I, while it gave two bands in the case of *Bam*HI. We have already obtained a cDNA clone for pumpkin mbAPX (Yamaguchi and Nishimura, unpublished data). The identity with amino acids between chloroplastic APXs and mbAPX is less than 30%. Although cDNA for pumpkin cAPX has not been cloned, comparison of cDNA sequences from another plants shows more than 80% identity between cAPXs and less than 30% identity between cAPX and chloroplastic APXs. These show that the probe used in Southern blotting (Fig. 3) does not hybridize to genes for mbAPX and cAPX under our experimental condition. These findings indicate that sAPX in pumpkin exists as a single-copy gene and alternative splicing produces two kinds of mRNAs for sAPX and tAPX.

RT-PCR was performed to monitor the presence of two kinds of mRNA as templates of first-strand cDNAs synthesized from mRNAs of 5-day dark- and 4-day light-grown cotyledons. The 5'-common sense primer of sAPX and tAPX (AsA3), and the 3'-specific antisense primers for tAPX (3'-non) and sAPX (SAPX-I) were used. Judging from the nucleotide sequences of sAPX (Fig. 2A) and tAPX [11], the combination of the AsA3 and 3'-non primers should give a 508-bp fragment and that of the AsA3 and SAPX-I primers should give a 406-bp fragment. These bands can be seen in lanes tAPX and sAPX of Fig. 4, respectively. Fig. 4 shows that each band is amplified in accordance with specific primers, suggesting that two kinds of mRNA are expressed within pumpkin leaves.

How are the expressions of sAPX and tAPX regulated? To obtain a clue, we investigated developmental changes in the levels of the sAPX and tAPX proteins in pumpkin cotyledons during germination and subsequent greening. We previously detected four APX isoenzymes with molecular masses of 38, 34, 31 and 28 kDa by using antibodies against pumpkin mbAPX [11]. Furthermore, it was shown from subcellular fractionation experiments that APX isoenzymes with the same molecular masses are localized in the thylakoid, stroma, microbodies and cytosol, respectively [11]. The relative amounts of two kinds of chloroplastic APXs (tAPX and sAPX) in the extracts of pumpkin cotyledons grown in the

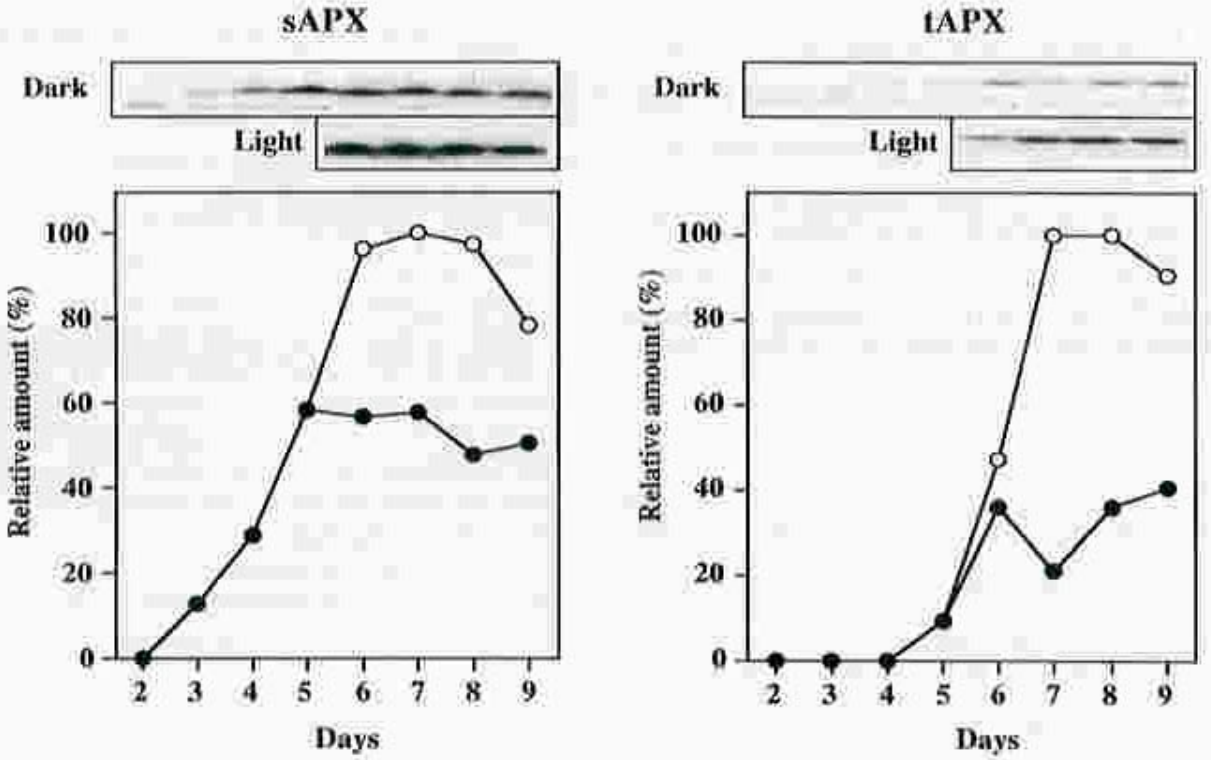


Fig. 5. Developmental changes in the relative amounts of sAPX and tAPX polypeptides in pumpkin cotyledons after germination. Total protein prepared from pumpkin cotyledons was subjected to SDS-PAGE, which was followed by immunodetection with antibodies against mbAPX (top panels). The relative amounts of the sAPX and tAPX polypeptides were determined densitometrically and plotted (bottom panels). (●) seedlings grown in darkness; (○) seedlings transferred to continuous illumination at the 5-day stage.

light and in the dark were determined by immunoblotting (Fig. 5, top panels). The immunoblot was quantified with a densitometer and plotted (Fig. 5, bottom panels). When seedlings were grown in darkness, the level of tAPX protein (right lower panel, closed circles) was hardly detected until the 5th day, whereas the level of sAPX protein (left lower panel, closed circles) gradually increased. The amounts of the two proteins increased rapidly when 5-day-old seedlings were transferred to an illuminated area (lower panels, open circles). Other chloroplastic enzymes also showed rapid increases as a result of illumination [22]. However, the different levels of induction of sAPX and tAPX in 7-day-old greening seedlings in the dark suggest that alternative splicing for sAPX and tAPX mRNAs in the cotyledons might be regulated by their developmental and environmental condition such as light. Although we performed this experiment several times with different extract preparations, the similar developmental patterns were obtained in all cases.

Alternative splicing is a well-known post-transcriptional regulatory mechanism in eukaryotic organisms. It has been reported that the synthesis of several enzymes in plants [15–20,23] and mammals [21,24,25] is controlled by alternative splicing. Previous work in our laboratory has shown that two kinds of cDNA clones for hydroxypyruvate reductase (HPR), HPR1 and HPR2, were produced by alternative splicing and their subcellular localizations were different because HPR1 protein contains the carboxy-terminal tripeptide Ser-Lys-Leu which is known as a targeting signal to microbodies [26,27] but the deduced HPR2 protein does not [17]. In this report, we provide another example that two proteins that are localized in different subcellular compartments and that are produced by alternative splicing. Further analysis at the mRNA level of sAPX and tAPX will provide new information on the regulatory mechanism of the expression of sAPX and tAPX by alternative splicing.

Acknowledgements: This work was supported by a grant for 'Research for the Future' Program (JSPS-RFTF 96L00407) from the Japan Society for the Promotion of Science and Grants-in-Aid for Scientific Research (No. 07456053, 04273101 and 04273102) from the Ministry of Education, Science and Culture, Japan, by a grant from the Nissan Science Foundation (Tokyo, Japan), by the NIBB program for Molecular Mechanisms of Stress Response.

References

[1] Yamaguchi, K., Mori, H. and Nishimura, M. (1995) Plant Cell Physiol. 36, 1157–1162.
[2] Bunkelmann, J.R. and Trelease, R.N. (1996) Plant Physiol. 110, 589–598.
[3] Koshiba, T. (1993) Plant Cell Physiol. 34, 713–721.
[4] Mittler, R. and Zilinskas, B.A. (1991) Plant Physiol. 97, 962–968.
[5] Chen, G.-X. and Asada, K. (1989) Plant Cell Physiol. 30, 987–998.
[6] Ishikawa, T., Sakai, K., Yoshimura, K., Takeda, T. and Shigeoka, S. (1996) FEBS Lett. 384, 289–293.
[7] Miyake, C., Cao, W.-H. and Asada, K. (1993) Plant Cell Physiol. 34, 881–889.
[8] Mittler, R. and Zilinskas, B.A. (1992) J. Biol. Chem. 267, 21802–21807.
[9] Mittler, R. and Zilinskas, B.A. (1994) Plant J. 5, 397–405.

[10] Chen, G.-X., Sano, S. and Asada, K. (1992) Plant Cell Physiol. 33, 109–116.
[11] Yamaguchi, K., Hayashi, M. and Nishimura, M. (1996) Plant Cell Physiol. 37, 405–409.
[12] Marchuk, D., Drumm, M., Saulino, A. and Collins, F.S. (1990) Nucl. Acids Res. 19, 1154–1154.
[13] Murray, M.G. and Thompson, W.F. (1980) Nucl. Acids Res. 8, 4321–4325.
[14] Laemmli, U.K. (1970) Nature 227, 680–685.
[15] Golovkin, M. and Reddy, A.S.N. (1996) Plant Cell 8, 1421–1435.
[16] Görlach, J., Raesecke, H.-R., Abel, G., Wehrli, R., Amrhein, N. and Schmid, J. (1995) Plant J. 8, 451–456.
[17] Hayashi, M., Tsugeki, R., Kondo, M., Mori, H. and Nishimura, M. (1996) Plant Mol. Biol. 30, 183–189.
[18] Kopriva, S., Cossu, R. and Bauwe, H. (1995) Plant J. 8, 435–441.
[19] Kopriva, S., Chu, C.-C. and Bauwe, H. (1996) Plant J. 10, 369–373.
[20] Tamaoki, M., Tsugawa, H., Minami, E., Kayano, T., Yamamoto, N., Kano-Murakami, Y. and Matsuoka, M. (1995) Plant J. 7, 927–938.
[21] Sajjadi, F.G., Boyle, D.L., Domingo, R.C. and Firestein, G.S. (1996) FEBS Lett. 382, 125–129.
[22] Gilmartin, P.M., Sarokin, L., Memelink, J. and Chua, N.-H. (1990) Plant Cell 2, 369–378.
[23] Theill, L.E., Hattori, K., Lazzaro, D., Castrillo, J.-L. and Karin, M. (1992) EMBO J. 11, 2261–2269.
[24] Hoemann, C.D., Bergeret, E., Guichard, A. and Griffin-Shea, R. (1996) Gene 168, 135–141.
[25] Sugiyama, T., Nishio, Y., Kishimoto, T. and Akira, S. (1996) FEBS Lett. 381, 191–194.
[26] Gould, S.J., Keller, G.-A. and Subramani, S. (1988) J. Cell Biol. 107, 897–905.
[27] Gould, S.J., Keller, G.-A., Hosken, N., Wilkinson, J. and Subramani, S. (1989) J. Cell Biol. 108, 1657–1664.

Light regulates alternative splicing of hydroxypyruvate reductase in pumpkin

Shoji Mano[1,2], Makoto Hayashi[1,2] and Mikio Nishimura[1,2,*]

[1] *Department of Cell Biology, National Institute for Basic Biology, Okazaki 444-0867, Japan*

[2] *Department of Molecular Biomechanics, School of Life Science, the Graduate University for Advanced Studies, Okazaki 444-0867, Japan*

Running title: Light-regulated alternative splicing

***Correspondent footnote:**
Dr. Mikio Nishimura; Department of Cell Biology, National Institute for Basic Biology, Okazaki 444, Japan
Phone : 81-564-55-7500
Fax : 81-564-55-7505
E-mail: mikosome@nibb.ac.jp

ABSTRACT

Hydroxypyruvate reductase (HPR) is a leaf peroxisomal enzyme that functions in the glycolate pathway of photorespiration in plants. We have obtained two high homologous cDNAs for pumpkin HPR (HPR1 and HPR2). It has been revealed that two HPR mRNAs might be produced by alternative splicing from a pre-mRNA. The HPR1 protein, but not the HPR2 protein, was found to have a targeting sequence into leaf peroxisomes at the carboxy terminus, suggesting that alternative splicing controls the subcellular localization of two HPR proteins. Immunoblot analysis and subcellular fractionation experiment showed that HPR1 and HPR2 proteins are localized in leaf peroxisomes and the cytosol, respectively. Moreover, indirect fluorescent microscopy and analyses of transgenic tobacco cultured cells and *Arabidopsis thaliana* expressing fusion proteins with green fluorescent protein (GFP) confirmed the different subcellular localizations of two HPR proteins. The amounts of both mRNAs were induced developmentally and by light, whereas each increase in quantity was different. Almost equal amounts of mRNAs were detected in pumpkin cotyledons grown in darkness, but treatment with light enhanced the much production of HPR2 mRNA. These findings indicate that light regulates alternative splicing of HPR mRNA, suggesting the presence of the novel mechanism of the mRNA maturation, namely light-regulated alternative splicing, in higher plants.

INTRODUCTION

Peroxisomes are ubiquitous organelles bound by a single membrane that exist in mammals, plants, fungi, yeast and invertebrates (2). In higher plants, peroxisomes are subcategorized functionally as glyoxysomes, leaf peroxisomes and unspecialized peroxisomes. Glyoxysomes, which are found in oil-rich tissues of etiolated cotyledons, contain some enzymes required for the β-oxidation of fatty acids and the glyoxylate cycle and play a major role in the mobilization of lipids that is necessary for the gluconeogenesis. Leaf peroxisomes, which are present in green leaves and cotyledons, contain enzymes of the glycolate cycle and participate in the photorespiration process. Unspecialized peroxisomes with undefined physiological function are present in other organs such as roots and stems. Titus and Becker (42) and our group (33) demonstrated that glyoxysomes are transformed directly to leaf peroxisomes during greening cotyledons of fatty seedlings, and that this transformation of peroxisomes is controlled by light. Degradation, translocation and gene expression of glyoxysomal enzymes are regulated in underlying transformation of peroxisomes from glyoxysomes to leaf peroxisomes. On the contrary, the reverse conversion from leaf peroxisomes to glyoxysomes occurs in the senescing cotyledons (7). In this case, leaf peroxisomal enzymes disappear and concomitantly glyoxysomal enzymes start to be accumulated. The reverse transformation of leaf peroxisomes to glyoxysomes was shown to occur directly (33). These reversible functional transformations of peroxisomes are unique organelle differentiation specifically observed in higher plants.

In order to clarify the regulatory mechanisms underlying these transformations of peroxisomes, a leaf peroxisomal enzyme, NADH-hydroxypyruvate reductase (HPR; EC 1. 1. 1. 29) was characterized. HPR catalyzes the conversion of hydroxypyruvate to glycerate. In higher plants, this enzyme is known to be localized in leaf peroxisomes, which are specialized peroxisomes found in photosynthetic tissues, and it plays a role in the glycolate pathway of photorespiration in concert with enzymes in chloroplasts and mitochondria (44). It has been reported that HPR activity and the amounts of its

transcripts are regulated both developmentally and by light (13). Similar results were obtained for glycolate oxidase, which is another leaf-peroxisomal enzyme (45). cDNAs for HPR have been cloned from pumpkin (16), cucumber (13), *Arabidopsis thaliana* (26) and methylotrophs (5, 50). In higher plants, HPR functions in the photorespiratory glycolate pathway, whereas in methylotrophs, it plays a role as an assimilatory enzyme of one-carbon compounds, such as methanol, in the serine pathway.

We previously reported that two kinds of cDNA clones for HPR (HPR1 and HPR2) were obtained from the cDNA library in pumpkin green cotyledons (16). The nucleotide sequences of these clones showed complete identity except for the region encoding the carboxy terminus. From the deduced amino acid sequences for HPR1 and HPR2, HPR1 protein has the carboxy terminal tripeptide of Ser-Lys-Leu which is known as a targeting signal to peroxisomes (11, 12, 15) but HPR2 protein does not, indicating that HPR1 and HPR2 proteins are localized in the peroxisomes and the cytosol, respectively. In addition, the genomic structure around this region showed the presence of two pairs of GT-AG doublets within the intron (16). From the comparison of the sequences of the genomic DNA with two cDNAs, it has been suggested that alternative splicing might give rise to two kinds of HPR mRNA. Namely, HPR1 mRNA is produced when the longer intron is spliced out, whereas HPR2 mRNA is produced when the 17-bp intron-like sequence, which has a stop codon, is retained. As a result, HPR1 protein might be transported into leaf peroxisomes due to the targeting signal at the carboxy terminus and HPR2 protein might remain in the cytosol on account of the lack for such a targeting sequence. Cucumber HPR cDNA, which is another registered HPR cDNA clone, does not have the carboxy terminal tripeptide (13). The HPR gene of cucumber also has the two pairs of GT-AG doublets at the similar position as that of pumpkin, suggesting the possibility that cucumber HPR also might be produced by alternative splicing, and that another HPR protein with a targeting signal to peroxisomes might exist in cucumber cells (16), although the cDNA has not been cloned.

Alternative splicing is a well-known post-transcriptional regulatory mechanism in eukaryotic organisms. The number of studies reporting on alternatively spliced genes in

higher plants has expanded in recent years (9, 14, 16, 19, 24, 27, 30, 38, 39, 41, 43). Interestingly, some of these enzymes showed different subcellular localizations (27, 30, 41). As stated above, it has been assumed that pumpkin HPRs are also localized in different parts of cells. In addition, as light regulates gene expression of HPR (13) and promotes the transformation of peroxisomes from glyoxysomes to leaf peroxisomes (34), it has been anticipated that light might regulate this alternative splicing. In this paper, we therefore tried to detect the two proteins and their mRNAs to investigate whether light takes part in alternative splicing of HPR mRNA, and to demonstrate two different subcellular localization within pumpkin cells.

MATERIALS AND METHODS

Plant materials. Pumpkin (*Cucurbita* sp. cv. Kurokawa Amakuri Nankin) seeds were soaked overnight and germinated in moist rock fiber (66R; Nitto Bouseki, Chiba, Japan) at 25 °C. Some of the seedlings were transferred to continuous light at the 5-day stage. Suspension-cultured tobacco cells (*Nicotiana tabaccum* L. cv Bright Yellow 2), kindly provided by Dr. K. Nakamura (Nagoya University), were maintained on a orbital shaker (Bio-Shaker BR-3000LF; TAITEC, Saitama, Japan) at 26.5 °C in the dark. Cells were subcultured once a week using Murashige-Skoog medium. Seeds of *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia were grown at 22 °C under continuous light.

Plasmid constructions and transformation into BY-2 and *Arabidopsis thaliana*. The constructs which were used to transform BY-2 and *Arabidopsis thaliana* were based on sGFP-TYG (6) which was kindly provided by Dr. Y. Niwa (University of Shizuoka). pSGFP-BE, which had a *Bam*H I site in the 5' flanking region and *Bgl* II and *Eco*R I sites on either side of the stop codon of *sGFP*, was generated by PCR using the following primers GFPsF (5'-CCGGATCCATGGTGAGCAAGGGCGAGGAG-3') and primer GFPsR (5'-GGGAATTCTCAGAGATCTCCCTTGTACAGCTCGTCCAT-3'). The fragment was subcloned into a T-vector prepared using pBluescript KS+ as described previously (28). In order to make the constructs for pMAT-SGFP-H1 and pMAT-SGFP-H2, the *Bgl* II-*Eco*R I fragment of the DNA fragment in pSGFP-BE was replaced with two kinds of double-stranded DNA produced by the annealing of complementary oligonucleotides. The complementary oligonucleotides were designed to produce protruding ends that ligate to the *Bgl* II and *Eco*R I sites of the *sGFP-BE* gene. The nucleotide sequences of the coding strands of the synthetic oligonucleotides used to construct chimeric genes are shown in Fig. 6. The *Bam*H I-*Eco*R I fragments that contain chimeric genes were inserted into the *Bgl* II-*Eco*R I site of a Ti-plasmid, pMAT037 (29).

The Ti-plasmids produced were then transformed into *Agrobacterium tumefaciens* (strain EHA101) by electroporation.

Transformation of BY-2 using *Agrobacterium tumefaciens* was performed according to the method of Matsuoka et al. (29).

Transformation of *Arabidopsis thaliana* (ecotype Columbia) was carried out by the infiltration method (1). Primary transformants were designated T0 plants. T1 seeds collected from T0 plants were surface sterilized in 2% NaClO plus 0.02% Triton X-100 and grown on germination media (2.3 μg ml^{-1} MS salts (Wako, Osaka, Japan), 1% sucrose, 100 μg ml^{-1} myo-inositol, 1 μg ml^{-1} thiamine-HCl, 0.5 μg ml^{-1} pyridoxine, 0.5 μg ml^{-1} nicotinic acid, 0.5 μg ml^{-1} MES-KOH (pH 5.7), 0.2% gellan gum (Wako, Osaka, Japan)) containing 100 μg ml^{-1} of kanamycin. T2 seeds were collected from approximately 10 independent T1 plants. T2 plants that accumulated the highest amount of transgene product were selected based on their fluorescence observed with fluorescent microscopy (see below).

Northern blot analysis. Ten μg of total RNA extracted from pumpkin cotyledons grown under various conditions was fractionated on a 1% gel that contained 0.66 M formaldehyde and 10 mM MOPS (pH 7.0). RNA was transferred to a Zeta-Probe blotting membrane (BIO-RAD, CA, USA) by the capillary method and fixed by exposure to UV-light (Funa-UV-Linker, model FS-800; Funakoshi, Tokyo, Japan). The fragment corresponding to pumpkin HPR cDNA was labeled with [^{32}P]-dCTP (Amersham Japan) using a Megaprime™ DNA labeling system (Amersham, Tokyo, Japan). The membrane was hybridized in 50% formamide, 0.12 M sodium phosphate (pH 7.2), 0.25 M sodium chloride, 7% SDS and 1mM EDTA (pH 8.0) with 1.0×10^6 cpm·ml^{-1} of radiolabeled DNA probe for 18 h at 42°C. The membrane was washed at 42°C in 2 × SSC plus 0.1% SDS for 15 min, in 0.2 × SSC plus 0.1% SDS for 15 min, in 0.1 × SSC plus 0.1% SDS for 15 min. The membrane was used to expose X-ray film and radioactivity was

measured on the imaging plate of a Bio-imaging analyzer (FUJIX BAS2000; FUJI Photo Film, Tokyo, Japan).

Immunoblot analysis. Pumpkin cotyledons grown under various stages were homogenized with the extraction buffer (100mM Tris-HCl, pH 6.8, 1mM EDTA, pH 8.0, 0.1% Triton X-100 and 1mM PMSF) and then the homogenates were centrifuged at 15,000 × *g* for 25 min. The protein content of each extract was estimated by using a protein assay kit (Nippon Bio-Rad Laboratories, Tokyo, Japan) with bovine gamma albumin as a standard protein. Ten μg of total protein was separated by SDS-PAGE on a 7.5% polyacrylamide gel as described by Laemmli (20) and transferred to a nylon membrane (Schleicher & Schuell, Dassel, Germany) in a semidry electroblotting system. Immunologic reactions were detected by monitoring the activity of horseradish peroxidase antibodies against rabbit IgG (ECL system; Amersham, Japan).

Southern blot analysis. Total DNA (5 μg) was digested with *Bam*H I, *Eco*R V, *Hin*c II, *Sma* I and *Sph* I. The products were fractionated on a 0.8% agarose gel, transferred to a Zeta-Probe blotting membrane (BIO-RAD, CA, USA) by the capillary method and fixed by exposure to UV-light (Funa-UV-Linker, model FS-800; Funakoshi, Tokyo, Japan). The fragment corresponding to pumpkin HPR cDNA was labeled with [^{32}P]-dCTP (Amersham Japan) using a Megaprime^{TM} DNA labeling system (Amersham, Tokyo, Japan). The conditions of hybridization and washing of the membrane were the same as previously described in Northern blot analysis. The membrane was used to expose X-ray film.

RT-PCR analysis. First-strand cDNA was generated using a Ready-To-Go T-primed first-strand kit (Pharmacia Biotech, Tokyo, Japan) from poly(A)$^+$ RNA templates

prepared from either 5-day dark- or 5-day dark- and 2-day light-grown cotyledons using Oligotex-dT 30 <Super> (Roche Japan, Tokyo, Japan). PCR was performed to amplify each HPR mRNA. The reaction mixture contained 1 unit of EX Taq DNA polymerase (Takara Shuzo, Kyoto, Japan), an upstream primer (N4; 5'-GCCGCTCTTAATGTTCTGGGA-3'), a downstream primer (B2C; 5'-TAATGCTTGCGATTGGTGCTG-3') and an appropriate buffer in a total volume of 50 µl. The condition was 25 cycles of 94 °C for 45 sec, 64 °C for 45 sec.

Subcellular fractionation. 5-day dark- and 2-day light-grown pumpkin cotyledons (2 g) were homogenized with razor blades for 5 min in 6 ml of chopping buffer (10mM Tricine-HCl, pH 7.5, 1mM EDTA and 0.5M sucrose). The homogenate was passed through four layers of cheesecloth. Then 1.5 ml of filtered sample was layered onto a sucrose gradient that consisted of a 1 ml cushion of 60% (w/w) sucrose and 11 ml of a linear sucrose gradient and was centrifuged at 25,000 × *g* for 3 h in an SW 28-1 rotor in an ultracentrifuge (model XL-90; Beckman, Fullerton, CA, USA). After centrifugation, fractions (0.5 ml each) were collected with an automatic liquid charger (ALC-2L; Advantec, Tokyo, Japan). All procedures were carried out at 4°C. Assays of HPR and catalase were carried out according to De Bellis et al. (7) and Yamaguchi and Nishimura (47), respectively.

Immunofluorescence staining of thin sections of pumpkin leaves. Pumpkin leaves were cut into 1-mm cubes and vacuum-infiltrated in a fixative (4% formaldehyde, 50 mM cacodylate buffer, pH 7.4, 60 mM sucrose, 0.001% Triton X-100). Fixed leaves were then incubated further in the same fixative for a further 2 h at 20 °C. Then the cubes were embedded in 5% agar. Thin sections (30 µm) were cut with razor blades on a Microslicer DTK-1000 (Dosaka, Japan). The sections were fixed on glass slides and treated with 0.1% pectolyase Y-23 for 2 h at 30 °C. Slides were washed three times with

excess PBS for 5 min each. The sections were further incubated with 0.3% Triton X-100 for 15 min at 20°C. Slides were washed three times with excess PBS for 5 min each and sections were treated with 5% BSA in PBS for 1 h at 20 °C. Then the sections were incubated with the first antibody for 1 h at 37 °C, washed three times with excess PBS for 5 min each and treated with 5% BSA in PBS for 30 min at 20 °C. The sections were incubated with 1:100 diluted FITC-conjugated goat antibodies against rabbit IgG and then washed three times with excess PBS for 5 min each.

Fluorescent microscopy. The photographs of fluorescence of the immunostained samples from pumpkin leaves and transgenic suspension-cultured tobacco cells were taken using an epifluorescence microscope (BHS2; Olympus, Tokyo, Japan) with a filter set (80P000 PINKEL 602, Photometrics, Japan) and Fujichrome 1600 film (Fuji Film, Tokyo, Japan).

Confocal microscopy. The immunostained pumpkin leaves and transgenic *Arabidopsis thaliana* were mounted in water under glass coverslips. The specimens were examined using a LSM-GB200 laser-scanning confocal microscope equipped with a krypton-argon laser and a filter set comprising BP535 and DM488 (Olympus, Tokyo, Japan) and using a TCS NT laser-scanning confocal microscope with a FITC filter set (Leica, Tokyo, Japan). Photographs were taken using Fujichrome 100 film (Fuji Film, Tokyo, Japan).

RESULTS

Pumpkin HPR is encoded by a single copy gene. In order to investigate whether a second HPR gene was present in pumpkin cells, we carried out a Southern blot analysis. Genomic DNA from pumpkin cotyledons was isolated, digested with *Bam*H I, *Eco*R V, *Hin*c II, *Sma* I and *Sph* I, and subjected to a Southern blot hybridization using pumpkin HPR cDNA as a probe. As shown in Fig. 1, HPR cDNA hybridized to single fragments when digested with *Bam*H I and *Sma* I, while it gave three, four or two bands when digested with *Eco*R V, *Hin*c II and *Sph* I, respectively. We cloned the HPR gene and checked the sites for restriction enzymes by digestion with the above restriction enzymes. As a result, there were two sites for *Eco*R V, three sites for *Hin*c II and one site for *Sph* I in the HPR gene (data not shown). Therefore, the bands shown in Fig. 1 seemed to have originated from internal sites for each restriction enzyme in the HPR gene. In addition, only one kind of genomic sequence was obtained when we amplified the genomic DNA (16), indicating that HPR in pumpkin exists as a single-copy gene, as it does in *Arabidopsis thaliana* (26) and cucumber (13).

Light affects patterns of alternatively spliced mRNA for HPR. The change in quantity of HPR mRNA at various developmental stages was investigated by Northern blot analysis (upper panel in Fig. 2A). The relative amounts of HPR mRNA were determined densitometrically after Northern blotting (lower panel in Fig. 2A). The amount of HPR mRNA was low until the 3-day stage and then increased gradually. When 5-day-old seedlings were exposed to continuous light, a dramatic increase in the level of mRNA was observed (open circles in Fig. 2A). However, when cotyledons were kept in darkness, the amount of mRNA reached the maximum level at 5 days after germination and then dropped rapidly (closed circles in Fig. 2A).

We could not detect two kinds of HPR mRNA by Northern blot hybridization, because the difference in length between two HPR mRNAs was only 17 nucleotides. In

order to resolve this problem, an RT-PCR analysis was carried out. As templates, first-strand cDNAs were synthesized from poly (A)$^+$RNAs prepared from either 5-day dark-grown or 5-day dark- and 2-day light-grown cotyledons. The 5'-sense primer (N4) and 3'-antisense primer (B2C) were used (Fig. 2B). Judging from the nucleotide sequences of two HPR cDNAs (16), the combination of N4 and B2C primers should give a 211-bp fragment for HPR1 and a 227-bp fragment for HPR2. Fig. 2C shows the results of one of several identical experiments. When each plasmid of HPR1 or HPR2 was used as a template, truncated fragments with different length of 17 nucleotides were amplified (lane 1 and 2 in Fig. 2C). Low amounts of two bands, which corresponded to HPR1 and HPR2 fragments, were detected when first-strand cDNA from the 5-day-old stage cotyledons was used as a template (lane 3 in Fig. 2C). In this case, the amounts of both fragments were almost the same. On the contrary, exposure to continuous light for 2 days enhanced the accumulation of HPR2 mRNA more than that of HPR1 mRNA (lane 4 in Fig. 2C), suggesting that light shifted the pattern for splicing of HPR to greater production of HPR2 mRNA.

Two kinds of HPR polypeptide are detected during development after seed germination. To identify the presence of two HPR proteins and examine the developmental changes of these polypeptides after germination, an immunoblot analysis was carried out using total extracts from pumpkin cotyledons under various stages. Fig. 3 shows that two electrophoretically close polypeptides with molecular masses of 42 kDa were recognized by antibodies against spinach HPR in the total extracts prepared from 7-day- and 9-day-old cotyledons. This is in good agreement with the molecular masses calculated from the deduced amino acids. Judging from the molecular masses of the HPR1 protein (42,305 Da) and HPR2 protein (41,709 Da), the upper and lower polypeptides must correspond to the HPR1 and HPR2 proteins, respectively, because only upper band exists in leaf peroxisomal fraction (see next section and Fig. 4).

With respect to the developmental changes of each polypeptide, the almost same amounts of both polypeptides increased gradually during germination in darkness. However, the abundances of the two polypeptides were induced markedly when seedlings were transferred to continuous light at the 5-day stage. In this case, the amount of HPR2 protein increased markedly than that of HPR1 protein. This results is in good agreement with the pattern of mRNAs (Fig. 2C). Densitometric analysis revealed that the ratio of HPR2 to HPR1 increased 1.0 to 2.4 when the seedlings were transferred to continuous light after 5 days.

Subcellular localization of two HPR proteins. As described previously, the deduced HPR1 protein contains a sequence of three amino acids, Ser-Lys-Leu, at the carboxy terminus that is known as a targeting signal to peroxisomes, while the deduced HPR2 protein does not have such a targeting sequence. This suggests that HPR1 and HPR2 proteins are localized in leaf peroxisomes and the cytosol, respectively. If this speculation is true, HPR activity should be detected in the cytosolic and peroxisomal fractions after subcellular fractionation of pumpkin cells. Accordingly, subcellular fractionation of pumpkin cotyledons by sucrose density gradient centrifugation was carried out. Odd fractions were subjected to immunoblotting analysis and the estimation of HPR activity. We also investigated catalase activity as a leaf-peroxisomal marker enzyme. As shown in Fig. 4, catalase activity was detected in the supernatant fractions which contained cytosolic enzymes (fraction nos. 1 and 3) as well as in the leaf-peroxisomal fraction (fraction no. 23), since leaf peroxisomes are so fragile that enzymes in the leaf peroxisomal matrix are released during homogenization and subcellular fractionation. The scales in Fig. 4 were adjusted so that the activities of HPR and catalase in the peroxisomal fraction (no. 23) were the same. If the percent of HPR that leaks from leaf peroxisomes is as same as the percent of catalase that leaks from leaf peroxisomes, and if HPR, like catalase, is exclusively localized in leaf peroxisomes, the activities of HPR and catalase in the supernatant fraction should be similar. However, the activity of

HPR in the cytosolic fraction was much higher than that of catalase. We speculated that this high activity originated from HPR2 protein in the cytosol in addition to the HPR1 protein that leaked from leaf peroxisomes. Antibodies against HPR certainly recognized the two polypeptides in the cytosolic fractions and one polypeptide in the peroxisomal fraction (lower panel in Fig. 4).

Moreover, indirect immunofluorescent analysis was performed to investigate the localizations of two HPR proteins in pumpkin cells. Cells isolated from pumpkin leaves were allowed to react with the antibodies against HPR or catalase, and the immunoreaction was visualized with FITC. As shown in Fig. 5, leaf peroxisomes were the only part in which well defined fluorescence was observed with the FITC filter after staining with catalase-specific antibodies (Fig. 5, A and B). Application of antibodies against HPR, however, revealed weak fluorescence throughout the entire cells in addition to strong spherical signals (Fig. 5, C and D). From the comparison with the case of catalase, these strong spherical signals represent HPR1 proteins in leaf peroxisomes and the weak fluorescence observed over the entire cell indicated the presence of cytosolic HPR2 protein.

Analysis of organelle targeting within transgenic BY-2 cells and root tissues of *Arabidopsis thaliana*. In order to confirm the subcellular localizations of the two HPR proteins, we constructed chimeric genes for fusion proteins containing green fluorescent protein (GFP) (Fig. 6). As stated above, the amino acid sequence of HPR1 and HPR2 proteins differs only in the carboxy terminal region. Hayashi et al. have reported that the carboxy terminal five amino acids of pumpkin malate synthase, a glyoxysomal enzyme, function as a targeting signal to peroxisomes such as glyoxysomes and leaf peroxisomes (15). We constructed two chimeric proteins consisting of GFP and the carboxy terminal ten residues of either HPR1 or HPR2 protein. Plasmid constructs were introduced into BY-2 cells (Fig. 7) and *Arabidopsis thaliana* (Fig. 8) via *Agrobacterium tumefaciens*. GFP without a targeting sequence, as a control, tends to

localize in the cytosol and on the surface of the nucleus (Fig. 7, C and Fig. 8, B and D), the results that are in good agreement with previous data (6). In the case of the chimeric protein with HPR1, well defined spots were observed to be distributed diffusely throughout the cytosol in BY-2 cells (Fig. 7, E) and in root tissues of *Arabidopsis thaliana* (Fig. 8, A and C). We concluded that the targeting sequence at the carboxy terminus of HPR1 directed GFP into peroxisomes in BY-2 cells and *Arabidopsis thaliana* root cells. On the contrary, the fluorescent pattern of chimeric protein with HPR2 demonstrated that it accumulated in the cytosol and on the surface of the nucleus (Fig. 7, G). These data confirm the results of indirect immunofluorescent analysis that HPR1 is transported into leaf peroxisomes because of the presence of the peroxisomal targeting signal, whereas HPR2 is retained in the cytosol because of the alternatively spliced elimination of such a targeting sequence.

DISCUSSION

Recent studies have demonstrated that some mRNAs in higher plants are also produced by alternative splicing like mammalian mRNAs are. Alternative splicing has a role in the regulation of gene expression and in the production of structurally and functionally different protein isoforms. Previous work in our laboratory indicated that mRNAs for pumpkin HPR might be produced by alternative splicing (16), although we did not obtained direct evidence of this. In this study, we were able to detect the presence of two kinds of HPR protein and mRNA. In addition, we showed that light controls this alternative splicing.

Two kinds of alternatively spliced HPR exist in different parts of pumpkin cells. According to our previous results, it is assumed that one of the two HPR proteins is transported into peroxisomes and the other is in the cytosol, because HPR1 protein, but not HPR2 protein, has the tripeptide of Ser-Lys-Leu at the carboxy terminus, which is known as a peroxisomal targeting signal 1 (PTS1). It has been reported that some glyoxysomal and peroxisomal enzymes have PTS1 at their carboxy termini (25, 45), and that the Ser-Lys-Leu sequence or its derivatives could direct reporter proteins such as β-glucuronidase (GUS) and chloramphenicol acetyltransferase (CAT) into peroxisomes (11, 12, 15). However, HPR2 protein lacks this PTS1 because of the presence of a stop codon within a 17-bp intron-like sequence produced by alternative splicing, suggesting that HPR2 protein is retained in the cytosol. Subcellular fractionation using sucrose density gradient centrifugation showed a higher activity for HPR in the cytosol than in leaf peroxisomes (upper panel in Fig. 4). Moreover, the polypeptide corresponding to HPR2 protein could be detected only in the cytosolic fraction (lower panel in Fig. 4). These results demonstrated the different localizations of HPR proteins within pumpkin cells. This result is supported by indirect immunofluorescent microscopic analysis (Fig. 5). From the comparison with the case of catalase, well defined spots and immunofluorescence over the cell represent the localizations of leaf peroxisomal and

cytosolic HPR, respectively. As mentioned previously, the amino acid sequences differ only in the carboxy terminal region. When each fusion protein, containing the carboxy terminal decapeptides of either HPR1 protein or HPR2 protein at the end of GFP, was expressed in BY-2 cells (Fig. 7) and *Arabidopsis thaliana* (Fig. 8), the fluorescence was observed to have different localizations. In addition, the Southern blot analysis showed that another HPR gene does not exist in the pumpkin genome (Fig. 1), demonstrating that the two HPR mRNAs do not originate from different genes.

Among higher plants, the small subunit of ADP-glucose pyrophosphorylase (AGPase) in barley, which is a key enzyme of starch biosynthesis, has different subcellular localizations produced by alternative splicing (41). In this case, alternative splicing produces two kinds of AGPase small subunit. One is transported into amyloplasts because of the presence of a transit peptide at the amino terminal region and the other remains in the cytosol since alternative splicing eliminates this transit peptide. Some studies have shown that both enzymes are involved in the production of ADP-glucose (4, 8, 40). This is the only case that the physiological role of each alternatively spliced protein has been characterized. In the case of HPR, HPR1 protein must play a role in photorespiration, but the physiological role of HPR2 protein remains to be determined. At present, the metabolic pathway necessary for HPR2 protein does not seem to be in the cytosol. HPR2 protein might catalyze the reduction of hydroxypyruvate which escapes from leaf peroxisomes, since the peroxisomal membrane might not be an absolute barrier to substrates and some overflow of hydroxypyruvate to the cytosol might be expected, especially at times when high light intensity activates the glycolate pathway. For example, Murray et al. discovered a high-CO_2-dependent barley mutant virtually devoid of peroxisomal HPR (32). When this mutant was allowed to assimilate labeled $^{14}CO_2$, the analysis of labeled products clearly demonstrated that substrate was passing through the photorespirate pathway, although there was an abnormal accumulation of label in serine. This result suggests that cytosolic HPR might be responsible for the reduction of hydroxypyruvate. In other higher plants, HPR activities in both leaf peroxisomes and the cytosol have been detected in spinach (18), pea leaf protoplasts (17)

and barley (32). But HPRs in spinach, pea leaf protoplast and barley seem to be derived from different genes by the following reasoning: (i) the molecular masses of leaf peroxisomal and cytosolic HPR are quite different, (ii) each HPR-specific antibody does not cross-react with each polypeptide, (iii) peroxisomal HPR uses NADH as the cofactor, whereas cytosolic HPR preferentially uses NADPH instead of NADH. When enzyme activity was measured with NADPH in place of NADH in pumpkin, activities were not detected in the leaf peroxisomal and cytosolic fractions (data not shown). This indicates that cytosolic HPR2 protein in pumpkin has different character from those in another plants.

Light modulates alternative splicing for HPR. We detected the same amounts of HPR1 and HPR2 proteins in the total extracts from dark-grown pumpkin cotyledons (Fig. 3). In darkness, the amounts increased gradually with time. However, the amounts of both proteins, especially HPR2, were induced dramatically by light, indicating that syntheses of two HPR proteins were regulated developmentally and by light, as is the case with another leaf peroxisomal enzyme, glycolate oxidase (45). Although HPR mRNA was also induced by light (Fig. 2A), we were unable to detect two kinds of mRNA corresponding to HPR1 mRNA and HPR2 mRNA by means of Northern blot hybridization because of the inability to resolve the HPR1 and HPR2 mRNAs due to their similar lengths. However, the RT-PCR analysis detected the two fragments corresponding HPR1 and HPR2 mRNAs, and revealed that the two HPR mRNAs were induced differently by light (Fig. 2C). Interestingly, in dark-grown cotyledons at the 5-day stage, the amounts of HPR1 and HPR2 mRNAs were almost the same (lane 3 in Fig. 2C). Whereas exposure to continuous light promoted a greater accumulation of HPR2 mRNA than HPR1 mRNA (lane 4 in Fig. 2C). This difference in the accumulation of mRNAs corresponds to the difference in the accumulation of the two HPR polypeptides (Fig. 3), suggesting that light effects splicing patterns of HPR pre-mRNA. That is, light changes alternative splicing from the production of almost equal amounts of HPR1 and

HPR2 mRNAs to mainly production of HPR2 mRNA. However, the function of HPR2 in the cytosol and the mechanism of production of much of the HPR2 mRNA are unknown. As another example of light-regulated alternative splicing, we have previously reported that alternative splicing of chloroplastic ascorbate peroxidase pre-mRNA was affected by the treatment with light (27). In this paper, we were able to present evidence that alternative splicing for HPR is also regulated by an environmental factor such as light in higher plants. In mammalians, gene expression that is controlled by alternative splicing shows a dependence on only the tissue type during development (31, 35, 37, 48, 51). However, light-regulated alternative splicing seems to be specific to plants, suggesting the presence of novel regulatory mechanisms on light response of higher plants.

The mechanism of alternative splicing has been reported in mainly mammalians to involve an exonic splicing enhancer (ESE) (49) and SR proteins acting as trans factors (46). ESEs are purine-rich sequences within exons and might be important as binding sites for a complex of proteins that are required for alternative splicing. We are currently trying to identify *cis*-elements in the HPR pre-mRNA that could be involved in alternative splicing. SR proteins have one or two RNA binding motifs at the amino terminal region and an arginine- and serine-rich domain at the carboxy terminal region (46). Cloning of the genes for mammalian SR proteins have shown that SR proteins occur in different molecular weights, and that the relative abundance of different SR proteins can influence alternative splicing that is related to tissue specificity (3, 10, 22, 36, 52), demonstrating that plants (tobacco and carrot) also possessed SR proteins. Several SR homologues have been cloned only in *Arabidopsis thaliana* and it has been shown that they could restore splicing in human S100 extracts that are deficient in SR protein, indicating that they play a crucial role in alternative splicing (21, 23). It might be possible that SR proteins are present in pumpkin cells and that they participate in regulation of alternative splicing for HPR. We are now trying to clone pumpkin SR proteins. The identification of *trans* factors and *cis*-elements will provide important information on the mechanism of alternative splicing in higher plants.

ACKNOWLEDGMENTS

We are most grateful to Dr. N. -H. Chua (Rockefeller University) for the antiserum against hydroxypyruvate reductase from spinach. We also thank Dr. Kenzo Nakamura (Nagoya University) for the gift of pMAT037 and Dr. Yasuo Niwa (University of Shizuoka) for providing cDNA of sGFP-TYG. This work was supported by a grant for "Research for the Future" Program (JSPS-RFTF 96L00407) from the Japan Society for the Promotion of Science and Grants-in-Aid for Scientific Research (Nos. 09440271, 09274101 and 09274103) from the Ministry of Education, Science and Culture, Japan and by the NIBB program for Molecular Mechanisms of Stress Response.

REFERENCES

1. **Bechtold, N., J. Ellis, and G. Pelletier.** 1993. *In planta Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. C. R. Acad. Sci. Paris, Life Sciences **316:**1194-1199.

2. **Beevers, H.** 1979. Microbodies in higher plants. Annu. Rev. Plant Physiol. **30:** 159-193.

3. **Chandler, S. D., A. Mayeda, J. M. Yeakley, A. R. Krainer, and X.-D. Fu.** 1996. RNA splicing specificity determined by the coordinated action of RNA recognition motif in SR proteins. Proc. Natl. Acad. Sci. USA **94:**3596-3601.

4. **Chen, B.-Y., and H. W. Janes.** 1997. Multiple forms of ADP-glucose pyrophosphorylase from tomato fruit. Plant Physiol. **113:**235-241.

5. **Chistoserdova, L. V., and M. E. Lidstrom.** 1994. Genetics of the serine cycle in *Methylobacterium extorquens* AM1: Identification of *sga*A and *mtd*A and sequences of *sga*A, *hpr*A and *mtd*A. J. Baceriol. **176:**1957-1968.

6. **Chiu, W.-l., Y. Niwa, W. Zeng, T. Hirano, H. Kobayashi, and J. Sheen.** 1996. Engineered GFP as a vital reporter in plants. Curr. Biol. **6:**325-330.

7. **De Bellis, L., and M. Nishimura.** 1991. Development of enzymes of the glyoxylate cycle during senescence of pumpkin cotyledons. Plant Cell Physiol. **32:**555-561.

8. **Denyer, K., F. Dunlap, T. Thorbjørnsen, P. Keeling, and A. M. Smith.** 1996. The major form of ADP-glucose pyrophosphorylase in maize endosperm is extra-plastidal. Plant Physiol. **112:**779-785.

9. **Golovkin, M., and A. S. N. Reddy.** 1996. Structure and expression of a plant U1 snRNP 70K gene: alternative splicing of U1 snRNP 70K pre-mRNAs produces two different transcripts. Plant Cell **8:**1421-1435.

10. **Gontarek, R. R., and D. Derse.** 1996. Interactions among SR proteins, an exonic splicing enhancer and a Lentivirus Rev protein regulate alternative splicing. Mol. Cell Biol. **16:**2325-2331.

11. **Gould, S. J., G.-A. Keller, N. Hosken, J. Wilkinson, and S. Subramani.** 1989. A conserved tripeptide sorts proteins to peroxisomes. J. Cell. Biol. **108:**1657-1664.

12. **Gould, S. J., G.-A. Keller, and S. Subramani.** 1988. Identification of peroxisomal targeting signals located at the carboxy terminus of four peroxisomal proteins. J. Cell. Biol. **107:**897-905.

13. **Greenler, J. M., J. S. Sloan, B. W. Schwartz, and W. M. Becker.** 1989. Isolation, characterization and sequence analysis of a full-length cDNA clone encoding NADH-dependent hydroxypyruvate reductase from cucumber. Plant Mol. Biol. **13:**139-150.

14. **Görlach, J., H.-R. Raesecke, G. Abel, R. Wehrli, N. Amrhein, and J. Schmid.** 1995. Organ-specific differences in the ratio of alternatively spliced chorismate synthase (*LeCS2*) transcripts in tomato. Plant J. **8:**451-456.

15. **Hayashi, M., M. Aoki, M. Kondo, and M. Nishimura.** 1997. Changes in targeting efficiencies of proteins to plant microbodies caused by amino acid substitutions in the carboxy-terminal tripeptide. Plant Cell Physiol. **38:**759-768.

16. **Hayashi, M., R. Tsugeki, M. Kondo, H. Mori, and M. Nishimura.** 1996. Pumpkin hydroxypyruvate reductases with and without a putative C-terminal signal for targeting to microbodies may be produced by alternative splicing. Plant Mol. Biol. **30:**183-189.

17. **Kleczkowski, L. A., C. V. Givan, J. M. Hodgson, and D. D. Randall.** 1988. Subcellular location of NADPH-dependent hydroxypyruvate reductase activity in leaf protoplasts of *Pisum sativum* L. and its role in photorespiratory metabolism. Plant Physiol. 88:1182-1185.

18. **Kleczkowski, L. A., and D. D. Randall.** 1988. Purification and characterization of a novel NADPH(NADH)-dependent hydroxypyruvate reductase from spinach leaves. Biochem. J. **250:**145-152.

19. **Kopriva, S., R. Cossu, and H. Bauwe.** 1995. Alternative splicing results in two different transcripts for H-protein of the glycine cleavage system in the C4 species *Flaveria trinervia*. Plant J. **8:**435-441.

20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during assembly of head of bacteriophage T4. Nature **227:**680-685.

21. **Lazar, G., T. Schaal, T. Maniatis, and H. M. Goodman.** 1995. Identification of a plant serine-arginine-rich protein similar to the mammalian splicing factor SF2/ASF. Proc. Natl. Acad. Sci. USA **92:**7672-7676.

22. **Lopato, S., A. Mayeda, A. R. Krainer, and A. Barta.** 1996. Pre-mRNA splicing in plants: Characterization of Ser/Arg splicing factors. Proc. Natl. Acad. Sci. USA **93:**3074-9079.

23. **Lopato, S., E. Waigmann, and A. Barta.** 1996. Characterization of a novel arginine/serine-rich splicing factor in Arabidopsis. Plant Cell **8:**2255-2264.

24. **Macknight, R., I. Bancroft, T. Page, C. Lister, R. Schmidt, K. Love, L. Westphal, G. Murphy, S. Sherson, C. Cobbett, and C. Dean.** 1997. *FCA*, a gene controlling flowering time in Arabidopsis, encodes a protein containing RNA-binding domains. Cell **89:**737-745.

25. **Mano, S., M. Hayashi, M. Kondo, and M. Nishimura.** 1996. cDNA cloning and expression of a gene for isocitrate lyase in pumpkin cotyledons. Plant Cell Physiol. **37:**941-948.

26. **Mano, S., M. Hayashi, M. Kondo, and M. Nishimura.** 1997. Hydroxypyruvate reductase with a carboxy-terminal targeting signal to microbodies is expressed in *Arabidopsis*. Plant Cell Physiol. **38:**449-455.

27. **Mano, S., K. Yamaguchi, M. Hayashi, and M. Nishimura.** 1997. Stromal and thylakoid-bound ascorbate peroxidases are produced by alternative splicing in pumpkin. FEBS Lett. **413:**21-26.

28. **Marchuk, D., M. Drumm, A. Saulino, and F. S. Collins.** 1990. Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. Nucleic Acids Res. **19:**1154-1154.

29. **Matsuoka, K., and K. Nakamura.** 1991. Propeptide of a precursor to a plant vacuolar protein required for vacuolar targeting. Proc. Natl. Acad. Sci. USA **88:**834-838.

30. **Mireau, H., D. Lancelin, and I. D. Small.** 1996. The same Arabidopsis gene encodes both cytosolic and mitochondrial alanyl-tRNA sythetases. Plant Cell **8:**1027-1039.

31. **Miyatake, R., A. Furukawa, M. Matsushita, K. Iwahashi, K. Nakamura, Y. Ichikawa, and H. Suwaki.** 1996. Tissue-specific alternative splicing of mouse brain type ryanodine receptor/calcium release channel mRNA. FEBS Lett. **395:**123-126.

32. **Murray, A. J. S., R. D. Blackwell, and P. J. Lea.** 1989. Metabolism of hydroxypyruvate in a mutant of barley lacking NADH-dependent hydroxypyruvate reductase, an important photorespiratory enzyme activity. Plant Physiol. **91:**395-400.

33. **Nishimura, M., Y. Takeuchi, L. D. Bellis, and I. Hara-Nishimura.** 1993. Leaf peroxisomes are directly transformed to glyoxysomes during senescence of pumpkin cotyledons. Protoplasma **175:**131-137.

34. **Nishimura, M., J. Yamaguchi, H. Mori, T. Akazawa, and S. Yokota.** 1986. Immunocytochemical analysis shows that glyoxysomes are directly transformed to leaf peroxisomes during greening of pumpkin cotyledon. Plant Physiol. **80:**313-316.

35. **Sajjadi, F. G., D. L. Boyle, R. C. Domingo, and G. S. Firestein.** 1996. cDNA cloning and characterization of A3i, an alternative spliced rat A3 adenosine receptor variant. FEBS Lett. **382:**125-129.

36. **Screaton, G. R., J. F. Cáceres, A. Mayeda, M. V. Bell, M. Plebanski, D. G. Jackson, J. I. Bell, and A. R. Krainer.** 1995. Identification and characterization of three members of the human SR family of pre-mRNA splicing factors. EMBO J. **14:**4336-4349.

37. **Si, K., K. Das, and U. Maitra.** 1996. Characterization of multiple mRNAs that encode mammalian translation initiation factor 5 (eIF-5). J. Biol. Chem. **271:**16934-16938.

38. **Sun, Y., B. A. Flannigan, J. T. Madison, and T. L. Setter.** 1997. Alternative splicing of cyclin transcripts in maize endosperm. Gene **195**:167-175.

39. **Tamaoki, M., H. Tsugawa, E. Minami, T. Kayano, N. Yamamoto, Y. Kano-Murakami, and M. Matsuoka.** 1995. Alternative RNA products from a rice homeobox gene. Plant J. **7**:927-938.

40. **Thorbjørnsen, T., P. Villand, K. Denyer, O.-A. Olsen, and A. M. Smith.** 1996. Distinct isoforms of ADP-glucose pyrophosphorylase occur inside and outside the amyloplasts in barley endosperm. Plant J. **10**:243-250.

41. **Thorbjørnsen, T., P. Villand, L. A. Kleczkowski, and O.-A. Olsen.** 1996. A single gene encodes two different transcripts for the ADP-glucose pyrophosphorylase small subunit from barley (*Hordeum vulgare*). Biochem. J. **313**:149-154.

42. **Titus, D. E., and W. M. Becker.** 1985. Investigation of the glyoxysome-peroxisome transition in germinating cucumber cotyledons using double-label immunoelectron microscopy. J. Cell. Biol. **101**:1288-1299.

43. **Tobias, C. M., and J. B. Nasrallah.** 1996. An S-locus-related gene in *Arabidopsis* encodes a functional kinase and produces two classes of transcripts. Plant J. **10**:523-531.

44. **Tolbert, N. E., A. Oeser, T. Kisaki, R. H. Hageman, and R. K. Yamazaki.** 1968. Peroxisomes from spinach leaves containing enzymes related to glycolate metabolism. J. Biol. Chem. **243**:5179-5184.

45. **Tsugeki, R., I. Hara-Nishimura, H. Mori, and M. Nishimura.** 1993. Cloning and sequencing of cDNA for glycolate oxidase from pumpkin cotyledons and Northern blot analysis. Plant Cell Physiol. **34**:51-57.

46. **Valcárcel, J., and M. R. Green.** 1996. The SR protein family: pleiotropic functions in pre-mRNA splicing. Trends Biochem. Sci. **21**:296-301.

47. **Yamaguchi, J., and M. Nishimura.** 1984. Purification of glyoxysomal catalase and immunochemical comparison of glyoxysomal and leaf peroxisomal catalase in germinating pumpkin cotyledons. Plant Physiol. **262**:261-267.

48. **Yang, T.-P., L. B. Agellon, A. Walsh, J. L. Breslow, and A. R. Tall.** 1996. Alternative splicing of the human cholesteryl ester transfer protein gene in transgenic mice. J. Biol. Chem. **271:**12603-12609.

49. **Yeakley, J. M., J.-P. Morfin, M. G. Rosenfeld, and X.-D. Fu.** 1996. A complex of nuclear proteins mediates SR protein binding to a purine-rich splicing enhancer. Proc. Natl. Acad. Sci. USA **93:**7582-7587.

50. **Yoshida, T., K. Yamaguchi, T. Hagishita, T. Mitsunaga, A. Miyata, T. Tanabe, H. Toh, T. Ohshiro, M. Shimao, and Y. Izumi.** 1994. Cloning and expression of the gene for hydroxypyruvate reductase (D-glycerate dehydrogenase) from an obligate methylotroph *Hyphomicrobium methylovorum* GM2. Eur. J. Biochem. **223:**727-732.

51. **Yu, Q., and B. P. Toole.** 1996. A new alternatively spliced exon between v9 and v10 provides a molecular basis for synthesis of soluble CD44. J. Biol. Chem. **271:**20603-20607.

52. **Zahler, A. M., W. S. Lane, J. A. Stolk, and M. B. Roth.** 1992. SR proteins: a conserved family of pre-mRNA splicing factors. Genes Dev. **6:**837-847.

FIGURE LEGENDS

Fig. 1. Southern blot analysis of pumpkin genomic DNA. Genomic DNA (5 μg each) was digested with the restriction endonucleases indicated, separated on a 0.8% agarose gel, transferred to a Zeta-Probe blotting membrane (BIO-RAD) and probed with a [^{32}P]-labeled pumpkin HPR cDNA fragment. The size of DNA fragments are shown in kbp on the left. B, *Bam*H I; E, *Eco*R V; H, *Hinc* II; Sm, *Sma* I; Sp, *Sph* I.

Fig. 2. Developmental changes in levels of HPR mRNA. (A) Northern blot analysis. Ten μg of total RNA extracted from pumpkin cotyledons grown under various conditions was separated on a 1% agarose gel. The RNA transferred to a Zeta-Probe membrane (BIO-RAD) was hybridized with a [^{32}P]-labeled cDNA fragment for pumpkin HPR. Radioactivity was measured densitometrically. (●) seedlings grown in darkness; (○) seedlings exposed to continuous illumination at the 5-day stage. (B) Schematic representation of cDNAs and the gene for pumpkin HPR. The hatched and filled boxes correspond to the two parts of a doublet intron. The filled box indicates a 17-bp intron-like sequence that is inserted into HPR2 cDNA. Primers for the RT-PCR, N4 and B2C, are shown with arrowheads indicating the polarity. (C) RT-PCR products for truncated HPR1 and HPR2 fragments detected on an agarose (1%)/NuSieve (3%) gel stained with ethidium bromide. Lanes were loaded with amplified cDNA derived from HPR1 cDNA (lane 1), HPR2 cDNA (lane 2), first-strand cDNA prepared from 5-day dark-grown pumpkin cotyledons (lane 3) and first-strand cDNA prepared from 5-day dark- and 2-day light-grown pumpkin cotyledons (lane 4).

Fig. 3. Developmental changes in levels of HPR1 and HPR2 polypeptides in pumpkin cotyledons after germination. Total protein prepared from cotyledons grown under various conditions was subjected to SDS-PAGE (10 μg each) which was followed by

immunodetection with antibodies against spinach HPR. HPR1 and HPR2 polypeptides are indicated by arrows, respectively. Lower photograph represents the immunodetection under shorter exposure time. Numbers above photographs represent the days after germination. 5-2 and 5-4 indicate cotyledons that were grown in the dark for 5 days and then kept in continuous light for 2 days or 4 days from the 5-day stage, respectively.

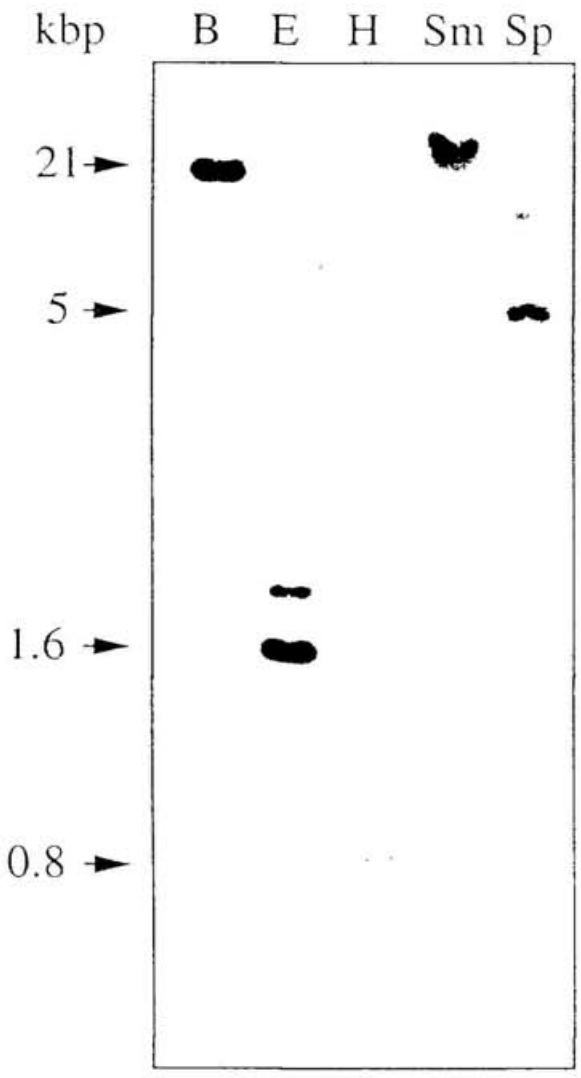
Fig. 4. Subcellular distribution of HPR protein in pumpkin cotyledons. Total protein prepared from 5-day dark- and 2-day light-grown pumpkin cotyledons was homogenized and the homogenate was subjected to sucrose density gradient centrifugation. After fractionation, activities of HPR and catalase were measured. HPR polypeptides were detected immunologically with antibodies against spinach HPR. (Upper panel) activities of HPR (■) and catalase (□). (Lower panel) 2% (v/v) of each fraction was subjected to SDS-PAGE (7.5% acrylamide). Arrows on the left represent the HPR1 and HPR2 polypeptides.

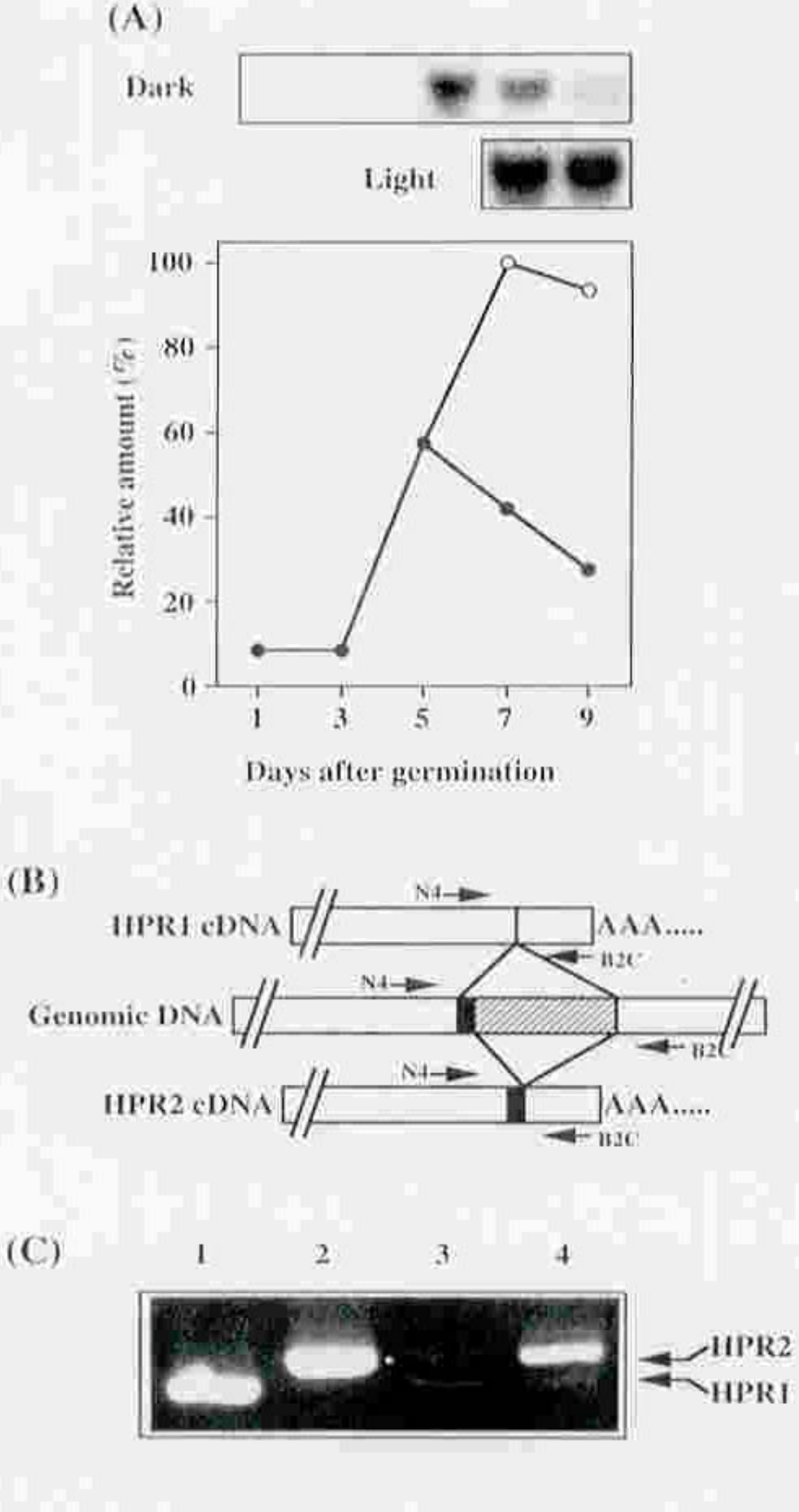
Fig. 5. Microscopic observations of pumpkin leaves stained with FITC. (A) and (B), immunofluorescent and confocal images, respectively, of thin sections of pumpkin leaf stained with catalase antibody and then with FITC-conjugated second antibody. (C) and (D), similar images except sections were first stained with HPR antibody. Bar indicates 10 μm.

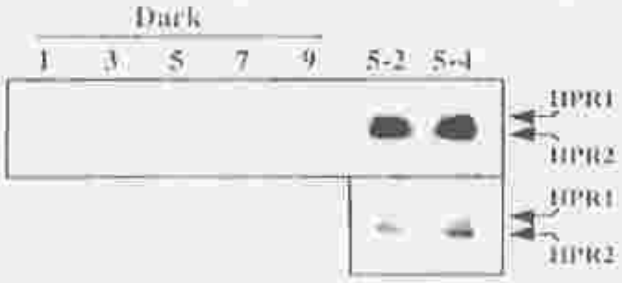
Fig. 6. Linear structures of chimeric constructs used in this study. The nucleotide sequences of DNA fragments inserted into the *Bgl* II-*Eco*R I site of pMAT-SGFP-BE are shown. The amino acid sequences are shown under the nucleotide sequences. The two amino acids shown in italics act as a linker between sGFP and each polypeptide. B, *Bam*H I; Bg, *Bgl* II; E, *Eco*R I

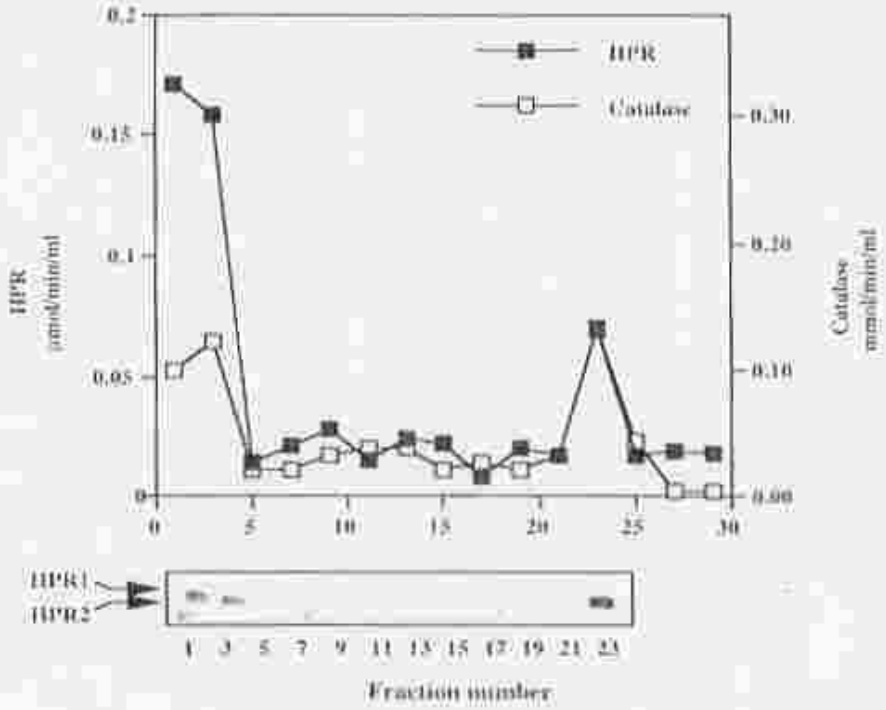
Fig. 7. Subcellular localizations of HPR proteins in transgenic tobacco BY-2 cells. BY-2 cells were observed in light field (Nomarski optics) and with blue light excitation (470-490 nm). (A) wild typed cells. (B) and (C) SGFP-BE. (D) and (E) SGFP-H1. (F) and (G) SGFP-H2. Bar indicates 10 μm.

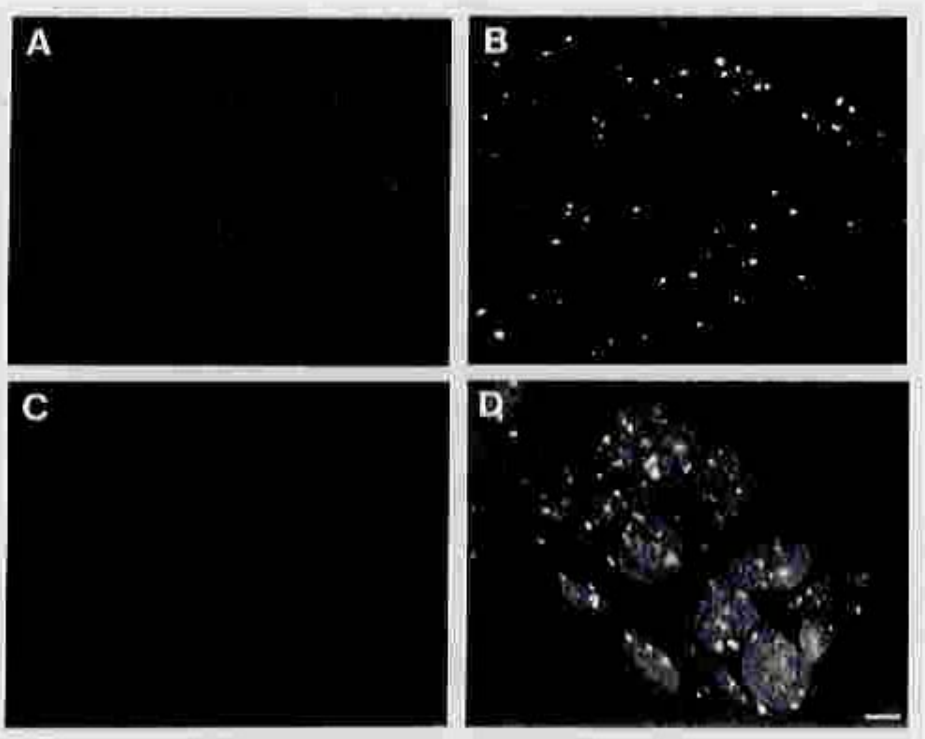
Fig. 8. Subcellular localizations of HPR proteins in root cells of transgenic *Arabidopsis thaliana*. Root cells in transgenic *Arabidopsis thaliana* were observed in light field (Nomarski optics) and with blue light excitation (470-490 nm). (A) and (B), 3-D images of SGFP-H1 and SGFP-BE in root tissues, respectively. (C) and (D), the same samples viewed with a confocal microscope. (E) and (F), the same samples viewed with a light microscope. Bar indicates 10 μm.


kbp
B
E
H
Sm
Sp
21
5
1.6
0.8


(A)
Dark
Light
100
80
60
40
20
0
Relative amount (%)
1
3
5
7
9
Days after germination
(B)
HPR1 cDNA
N4
AAA.....
B2C
Genomic DNA
N4
B2C
HPR2 cDNA
N4
AAA.....
B2C
(C)
1
2
3
4
HPR2
HPR1


Dark
1
3
5
7
9
5-2
5-4
HPR1
HPR2
HPR1
HPR2


HPR
Catalase
0.2
0.15
0.1
0.05
0
0.30
0.20
0.10
0.00
0
5
10
15
20
25
30
HPR activity (nmol/min/ml)
Catalase (nmol/min/ml)
HPR1
HPR2
1 3 5 7 9 11 13 15 17 19 21 23
Fraction number


A
B
C
D

GATCTCAAAGCCTTGGAATTGCCTGTGTCAAAGCTGTG

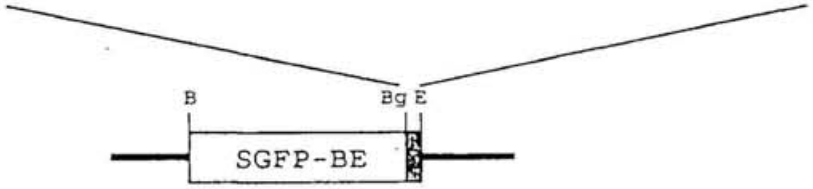
pMAT-SGFP-H1 AGTTTCGGAACCTTAACGGACACAGTTTCGACACTTAA

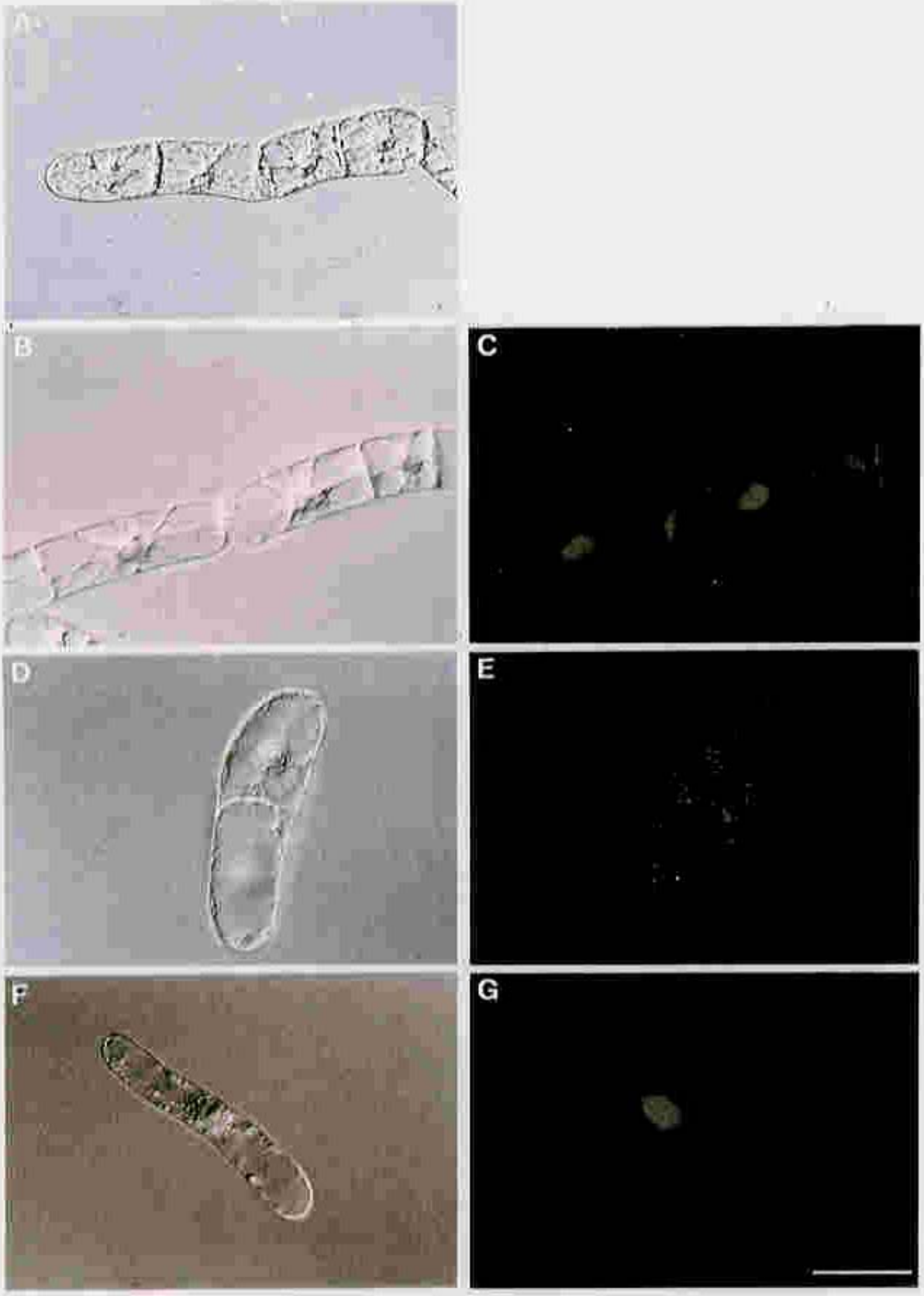
D L K A L E L P V S K L *

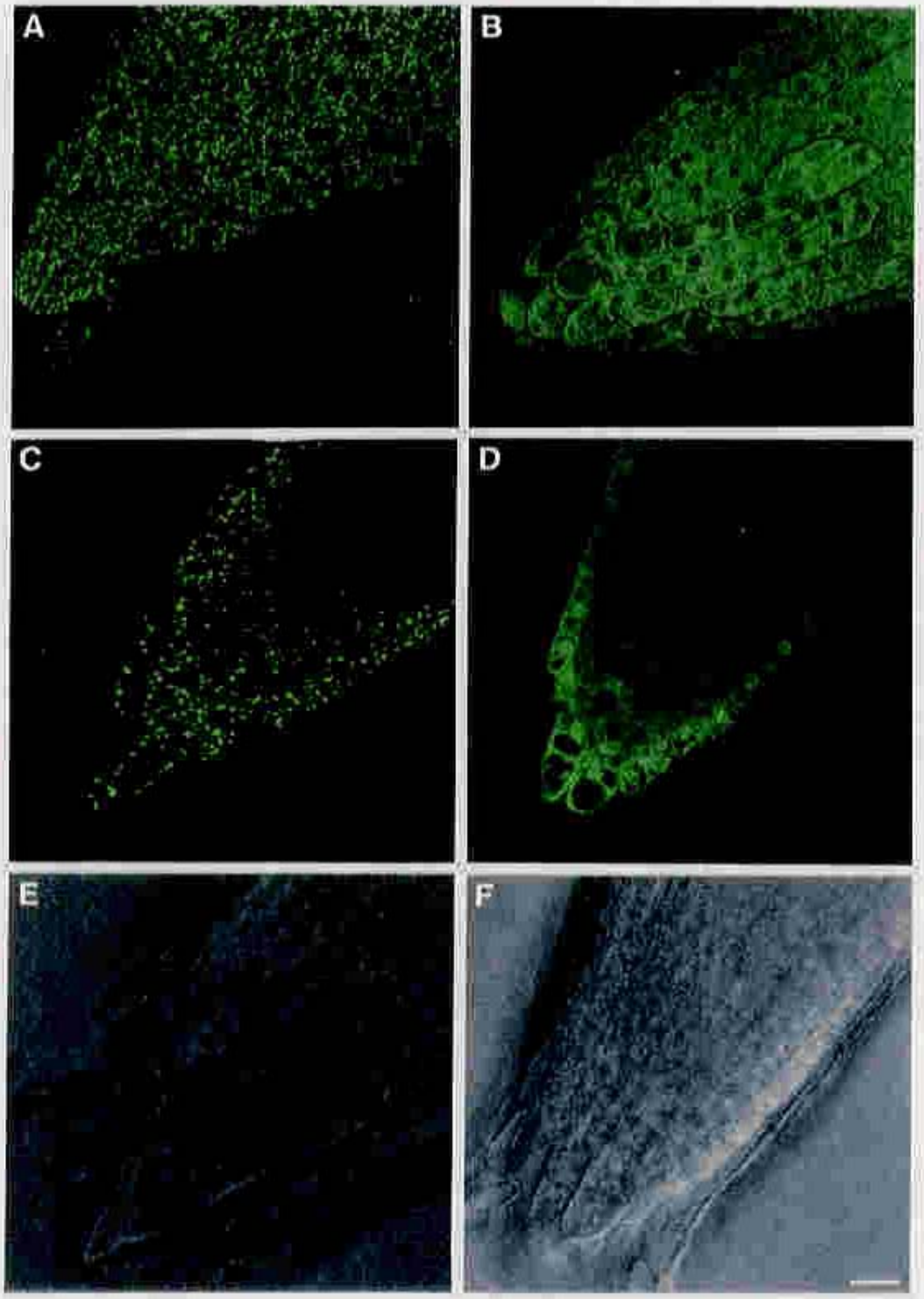
GATCTCAGCATTGTGAATGCAAAAGCCTTGGGTATATAAG

pMAT-SGFP-H2 AGTCGTAACACTTACGTTTTCGGAACCCATATATTCTTAA

D L S I V N A K A L G I *




A
B
C
D
E
F
G


A
B
C
D
E
F

Reviews and other publication

Shin Takeda, Shoji Mano, Masa-aki Ohto and Kenzo Nakamura (1994) Inhibitors of protein phosphatases 1 and 2A block the sugar-inducible gene expression in plants. *Plant Physiol.* **106**: 567-574.

Shoji Mano, Makoto Hayashi, Maki Kondo and Mikio Nishimura (1996) cDNA cloning and expression of a gene for isocitrate lyase in pumpkin cotyledons. *Plant Cell Physiol.* **37**: 941-948.

Mikio Nishimura, Makoto Hayashi, Akira Kato, Katsushi Yamaguchi and Shoji Mano (1996) Functional transition of microbodies in higher plants. *Cell Struc. Func.* **21**: 387-393.

Shoji Mano (1997) The analysis of protein transport using GFP: II. A method for suspend cultured cells. A separate number of Cell Technology (Plant Cell Technology Volume **6**: Experimental procedures for the observation of plant cells). Edited by Hiroo Fukuda, Mikio Nishimura and Kenzo Nakamura. pp. 125-129. SHUJUNSHA Co., Ltd. press, Tokyo. (in Japanese).

Plant Physiol. (1994) 106: 567–574

Inhibitors of Protein Phosphatases 1 and 2A Block the Sugar-Inducible Gene Expression in Plants[1]

Shin Takeda, Shoji Mano, Masa-aki Ohto, and Kenzo Nakamura*

Laboratory of Biochemistry, School of Agriculture, Nagoya University, Chikusa, Nagoya 464-01, Japan

Genes coding for two major proteins of the tuberous root of sweet potato (*Ipomoea batatas*), namely, sporamin and β-amylase, are inducible in leaves and petioles when they are supplied with high concentrations of sucrose or other metabolizable sugars, such as glucose and fructose, and the accumulation of a large amount of starch accompanies this induction. Three inhibitors of protein phosphatases 1 (PP1) and 2A (PP2A), namely, okadaic acid, microcystin-LR, and calyculin A, strongly inhibited the sucrose-inducible accumulation of mRNAs for sporamin, β-amylase, and the small subunit of ADP-glucose pyrophosphorylase in petioles. However, these inhibitors did not have any major effect on the steady-state levels of mRNAs for catalase and glyceraldehyde-3-phosphate dehydrogenase, and the sucrose-inducible increase in the level of sucrose synthase mRNA was enhanced by okadaic acid. Inhibitors of PP1 and PP2A also inhibited sucrose-inducible expression of a fusion gene, consisting of the promoter of the sweet potato gene for β-amylase and the coding sequence for β-glucuronidase (GUS), in leaves of transgenic tobacco (*Nicotiana tabacum*). The inhibition was not due to inhibition of uptake and cleavage of sucrose, since okadaic acid also inhibited induction of the fusion gene by glucose or fructose. Addition of okadaic acid to leaves that had been treated with sucrose for 6 h inhibited further increases in GUS activity. These results suggest that the continuous dephosphorylation of proteins is required in the transduction of carbohydrate metabolic signals to the transcriptional activation of at least some sugar-inducible genes in plant.

During the growth of many plant species, sugars synthesized in photosynthetic cells are transported to nonphotosynthetic cells and tissues in the form of Suc. In young, developing plants, most of the sugars are synthesized in source leaves and transported to sites where there is a demand for carbohydrate to support growth. However, in maturing plants, most of the sugars are transported to reproductive storage organs, such as seeds, tubers, and fruits for storage as storage polysaccharides or lipids. Some of the genes coding for storage proteins and starch-biosynthetic enzymes in the vegetative storage organs of plants such as potato and sweet potato are under metabolic regulation by carbohydrates, and they are induced by high levels of Suc or other metabolizable sugars (Rocha-Sosa et al., 1989; Wenzler et al., 1989; Hattori et al., 1990, 1991; Müller-Röber et al., 1990; Nakamura et al., 1991). The induction of expression of these genes by sugars is regulated at the level of transcription and can be documented in transgenic plants by fusion of a promoter with a reporter gene (Rocha-Sosa et al., 1989; Wenzler et al., 1989; Ohta et al., 1991). Sugar-inducible or sugar-modulated expression has also been reported for several other plant genes (Salanoubat and Belliard, 1989; Johnson and Ryan, 1990; Tsukaya et al., 1991; Cheng et al., 1992; Koch et al., 1992). Furthermore, several plant genes that are down-regulated by high levels of sugars have also been reported (Sheen, 1990; Yu et al., 1991; Koch et al., 1992). These results suggest that the metabolic regulation of gene expression by sugar availability is a general phenomenon in many plant species. However, the mechanisms underlying such regulation, in particular the mechanisms of signal transduction, are unknown at present.

In sweet potato (*Ipomoea batatas*), genes coding for two major proteins of the tuberous storage root, sporamin and β-amylase, are inducible in leaves and petioles when leaf-petiole cuttings are supplied with high concentrations of Suc or other metabolizable sugars, such as Glc and Fru (Hattori et al., 1991; Nakamura et al., 1991). The accumulation of large amounts of starch occurs concomitantly with the induction of sporamin and β-amylase by sugars (Nakamura et al., 1991). The physiological role of β-amylase in sweet potato is not known, and we previously postulated that it may have a role in part as a storage protein (Nakamura et al., 1991; Ohto et al., 1992). Sugar-inducible gene expression is probably associated with the high-level accumulation of sporamin, β-amylase, and starch in tuberous roots, which develop as a strong carbohydrate sink (Hattori et al., 1991; Nakamura et al., 1991).

In the present study, we examined whether the dephosphorylation of proteins is involved in the sugar-inducible expression of the genes for sporamin and β-amylase by using okadaic acid, microcystin-LR, and calyculin A. These three compounds are potent and specific inhibitors of protein-Ser/Thr phosphatases 1 and 2A, and they have broad and overlapping substrate specificities (Ishihara et al., 1989; Cohen et al., 1990; MacKintosh et al., 1990). In plants, PP1 and PP2A are known to regulate various enzymatic activities in vitro

[1] This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas ("Plasticity and Signal Responses in Plant Gene Expression") from the Ministry of Education, Science, and Culture, Japan.

* Corresponding author; fax 81-52-789-4094.

Abbreviations: AGPase, ADP-Glc pyrophosphorylase; β-Amy:GUS, a fusion gene composed of the promoter of the sweet potato gene for β-amylase and the coding sequence of β-glucuronidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GUS, β-glucuronidase; PP1, type 1 protein phosphatase; PP2A, type 2A protein phosphatase.

(Carter et al., 1990; Siegl et al., 1990; MacKintosh et al., 1991; Huber and Huber, 1992; Huber et al., 1992), and inhibitors of PP1 and PP2A were recently used to examine the involvement of protein phosphatases in the responses of plant genes to light (Sheen, 1993), phytohormones (Dominov et al., 1992; Raz and Fluhr, 1993), and fungal elicitors (MacKintosh et al., 1994) in vivo.

The results presented in this report indicate that, in sweet potato, inhibitors of PP1 and PP2A strongly and specifically block the Suc-inducible expression not only of genes for sporamin and β-amylase but also of a gene for the small subunit of AGPase, which is a key enzyme in starch biosynthesis. Inhibitors of PP1 and PP2A also blocked the induction of expression of β-Amy:GUS in leaves of transgenic tobacco by exogenous supply of Suc, Glc, or Fru. These results suggest that protein dephosphorylation is involved in the transduction of carbohydrate metabolic signals to the transcriptional activation of gene expression in plants.

MATERIALS AND METHODS

Materials

Inhibitors of protein phosphatases (okadaic acid and calyculin A from Wako Pure Chemical Industries, Osaka, Japan, and microcystin-LR from Calbiochem, San Diego, CA) were dissolved in 1 mM DMSO and stored at −20°C. Sweet potato (*Ipomea batatas* Lam. cv Kokei No. 14) plants were grown at Nagoya University Experimental Farm. Transgenic tobacco (*Nicotiana tabacum* cv SR1) plants carrying a β-Amy:GUS fusion gene, composed of a 1.3-kb-long region of the promoter of the gene for β-amylase from sweet potato (Yoshida et al., 1992) and the coding sequence for GUS (Jefferson et al., 1987), were prepared by the *Agrobacterium*-mediated transformation. Construction and expression of the fusion gene in various tissues of transgenic tobacco and the analysis of the promoter fragment will be described elsewhere (S. Ishiguro, K. Hayashi, K. Maeo, A. Morikami, and K. Nakamura, unpublished data). Seeds of transgenic tobacco plants were germinated on basal medium that contained Murashige-Skoog salts, B5 vitamins, 3% Suc, and 200 mg/L kanamycin, and seedlings were grown on the same medium supplemented with 50 mg/L kanamycin (Ohta et al., 1991). After transfer to soil, plants were grown to average heights of about 40 cm.

Treatment of Plant Tissues with Suc and Inhibitors of PP1 and PP2A

Mature leaves were excised from stems of sweet potato plants at their petioles. These leaf-petiole cuttings were dipped at their cut edges in a solution of 6% Suc and incubated in this way in darkness for 12 h (Ohto et al., 1992). In the case of treatment with inhibitors of PP1 and PP2A, leaf-petiole cuttings were pretreated with inhibitors in 0.2% DMSO for 2 h before the treatment with Suc, and inhibitors were also added to the Suc solution. After treatments, petiole portions of the cuttings were frozen in liquid nitrogen and stored at −80°C.

Fully expanded leaves of transgenic tobacco plants carrying the β-Amy:GUS fusion gene were cut into pieces of about 1 cm^2, and these leaf segments were incubated on a 0.4% agar plate that contained 10% Suc in darkness for 20 h. In some experiments, the agar plate contained 0.3 M Glc or 0.3 M Fru instead of Suc. Inhibitors of PP1 and PP2A were added to the agar plate with 0.05% DMSO.

Northern Blot Hybridization

Preparation of total RNA from sweet potato petioles and northern blot hybridization with ^{32}P-labeled cDNA probes were carried out as described previously (Nakamura et al., 1991). The cDNA probes for sporamin (Hattori et al., 1985), β-amylase (Yoshida and Nakamura, 1991), and catalase (Sakajo et al., 1987) from sweet potato were described previously. Probes for the small subunit of AGPase and Suc synthase were prepared by reverse transcriptase-PCR from poly(A)$^+$ RNA isolated from Suc-treated petioles of sweet potato. Amplified DNA fragments were cloned in a plasmid vector and verified by DNA sequencing. A cDNA fragment for the small subunit of AGPase encoded a protein with 96% amino acid residual identity with the corresponding part of the potato subunit (Nakata et al., 1991), and it did not cross-hybridize with a cDNA fragment for the large subunit of AGPase of sweet potato isolated by the similar methods. A cDNA for GAPDH mRNA from rice was a generous gift from Dr. A. Kato of Hokkaido University and Dr. H. Uchimiya of Tokyo University. Autoradiography was carried out by exposing washed membranes to x-ray film (X-Omat, Eastman Kodak, Rochester, NY). Alternatively, the radioactivity in bands was quantified with a Bio-Imaging Analyzer (Fujix BAS2000; Fuji Photo Film Co., Tokyo, Japan).

Extraction of Proteins and Assay of GUS Activity

Extraction of proteins from the leaf segments of transgenic tobacco plants and fluorimetric assays of GUS activity were carried out as described by Jefferson et al. (1987).

RESULTS

Inhibitors of PP1 and PP2A Inhibit the Suc-Inducible Accumulation of mRNAs for Sporamin, β-Amylase, and the Small Subunit of AGPase in Petioles of Sweet Potato

To analyze the effects of specific inhibitors of PP1 and PP2A on the sugar-inducible accumulation of mRNAs, leaf-petiole cuttings of sweet potato were treated with 6% Suc in the presence of 2 μM okadaic acid, microcystin-LR, or calyculin A and also in their absence. Total RNA prepared from the petiole portions was analyzed by northern blot hybridization in each case. As shown in Figure 1, a marked increase in levels of mRNAs for both sporamin and β-amylase occurred in petioles that had been treated with 6% Suc for 12 h but not in those treated with water as reported previously (Nakamura et al., 1991; Ohto et al., 1992). Treatment of the leaf-petiole cuttings with Suc also induced the accumulation of a large amount of starch (Nakamura et al., 1991). Northern blot hybridization analysis with the ^{32}P-labeled probe for the small subunit of AGPase (Nakata et al., 1991), a key enzyme in the biosynthesis of starch, indicated that mRNA for the small subunit of AGPase is also induced after treatment with

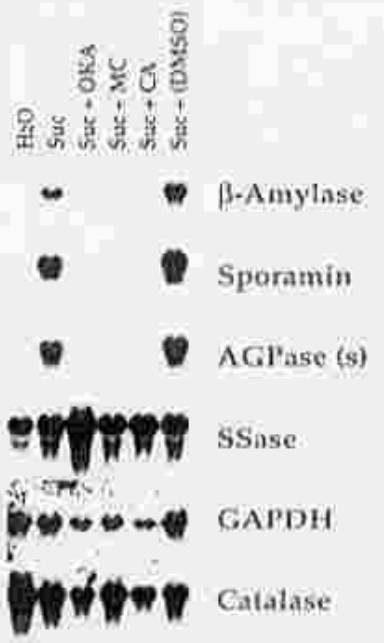


Figure 1. Northern blot hybridization analysis of mRNAs from petiole tissues of sweet potato after treatment with Suc in the presence of inhibitors of PP1 and PP2A. Leaf-petiole cuttings were treated with 6% Suc for 12 h in the presence of 2 μM okadaic acid (Suc + OKA), 2 μM microcystin-LR (Suc + MC), or 2 μM calyculin A (Suc + CA), dissolved in 0.2% DMSO or in their absence (Suc + (DMSO)) as described in "Materials and Methods." Leaf-petiole cuttings treated with 0% (H_2O) and 6% Suc (Suc) for 12 h were also prepared. Total RNA from petiole tissue (20 μg) were applied to each lane of the gel, and after electrophoresis bands of RNA were blotted onto membranes. Membranes were hybridized with the indicated ^{32}P-labeled cDNA probes. SSase, Suc synthase.

Suc (Fig. 1). All of the inhibitors of PP1 and PP2A examined almost completely inhibited the Suc-induced accumulation of mRNAs for sporamin, β-amylase, and the small subunit of AGPase. Similar results were obtained in several separate experiments.

The inhibitory effects were not due to the 0.2% DMSO that was present in the solution of Suc to dissolve the inhibitors. Addition of 0.2% DMSO to the solution of Suc slightly enhanced the levels of mRNAs (Fig. 1), whereas treatment with 0.2% DMSO alone did not cause induction of mRNAs (data not shown). To examine the specificity of the effects of the inhibitors, we allowed the RNA blot membranes to hybridize with several other cDNA probes. The levels of mRNAs for GAPDH and catalase were not significantly affected by the inhibitors, although a slight decrease was observed in some experiments (Fig. 1). Treatment of the tissues with Suc caused an increase in the level of mRNA for Suc synthase. Sugar-modulated changes in the level of Suc synthase mRNA were observed previously in potato and maize (Salanoubat and Belliard, 1989; Koch et al., 1992). In contrast to the decreases in levels of mRNAs for sporamin, β-amylase, and the small subunit of AGPase, the Suc-induced increase in the level of Suc synthase mRNA was significantly enhanced by 2 μM okadaic acid (Fig. 1). The enhancement of the level of Suc synthase mRNA by microcystin-LR and calyculin A after treatment of tissues with Suc was not as marked as that observed with okadaic acid. These results indicate that the inhibitors do not affect general transcriptional activity or the stability of mRNAs under these conditions and that their effects on mRNAs for sporamin, β-amylase, and the small subunit of AGPase are specific.

We examined the effects of lower concentrations of inhibitors on the Suc-induced changes in the levels of various mRNAs. Table I shows the relative amounts of mRNAs from petioles that had been treated with Suc in the presence of 0, 0.02, 0.2, and 2 μM okadaic acid and microcystin-LR for 12 h. The amount of radioactive probe that hybridized to each mRNA in the sample of RNA from petioles that had been treated with Suc in the absence of inhibitor was assigned a

Table I. *Effects of various concentrations of okadaic acid and microcystin-LR on the levels of various mRNAs in petiole tissues of sweet potato after treatment with Suc.*

Leaf-petiole cuttings were treated with 6% Suc in the presence of various concentrations of okadaic acid (OKA) or microcystin-LR (MC) for 12 h, and the levels of various mRNAs were analyzed as described in Figure 1. The radioactivity of each band, relative to the amount of respective mRNA in the control cuttings that had been incubated without inhibitors, is shown. For abbreviations, see legend to Figure 1.

Treatment	Relative Amount of mRNA					
	β-Amylase	Sporamin	AGPase(s)	SSase	GAPDH	Catalase
Suc + (DMSO)	1.00	1.00	1.00	1.00	1.00	1.00
Suc + 0.02 μM OKA	0.64	0.57	0.81	0.82	1.01	1.20
Suc + 0.2 μM OKA	0.29	0.40	0.53	0.90	1.01	1.14
Suc + 2 μM OKA	0.10	0.26	0.13	2.44	0.86	0.68
Suc + 0.02 μM MC	1.38	1.09	1.01	1.01	0.99	1.16
Suc + 0.2 μM MC	0.94	0.79	0.82	0.77	1.03	1.35
Suc + 2 μM MC	0.10	0.18	0.22	1.06	0.82	1.21

unit of 1. Okadaic acid had inhibitory effects on the Suc-induced accumulation of mRNAs for sporamin, β-amylase, and the small subunit of AGPase even at concentrations of 0.02 and 0.2 μM (Table I). At these concentrations, okadaic acid did not significantly affect the levels of other mRNAs, such as those for GAPDH, catalase, and Suc synthase. Similar to the results shown in Figure 1, 2 μM okadaic acid significantly enhanced the Suc-induced accumulation of Suc synthase mRNA. Microcystin-LR at 0.02 and 0.2 μM only slightly inhibited the Suc-induced accumulation of mRNAs for sporamin, β-amylase, and the small subunit of AGPase, as compared to the strong inhibitory effects observed at 2 μM (Table I). Microcystin-LR did not change the levels of other mRNAs significantly.

Effects of Inhibitors of Protein Phosphatases on the Sugar-Inducible Expression of the β-Amy:GUS Fusion Gene in Leaves of Transgenic Tobacco

Sugar-inducible gene expression can be easily assayed by measuring the GUS activity in leaf segments of transgenic tobacco plants that have been transformed with a β-Amy:GUS fusion gene, in which a 1.3-kb-long promoter fragment from the sweet potato gene for β-amylase (Yoshida et al., 1992) is fused to the GUS-coding sequence. When leaf segments were placed on agar plates that contained 10% Suc and incubated in darkness, a marked increase in GUS activity was induced. By using this system, we asked whether the effects of phosphatase inhibitors on the sugar-inducible expression of the gene for β-amylase occurred at the level of its transcription and whether the involvement of protein phosphatases is a general feature of sugar-inducible gene expression in plants. Furthermore, unlike the case of petiole tissues of sweet potato, to which Suc and inhibitors were supplied by the transpiration stream from the cut edges of leaf-petiole cuttings, Suc and inhibitors were directly supplied to leaf tissues of tobacco.

Leaf segments from one transgenic plant were divided into several groups, with each group consisting of six segments, and each group was treated with 10% Suc in the presence or absence of various inhibitors of PP1 and PP2A for 20 h. The specific activity of GUS in protein extracts from each group of leaf segments was expressed relative to that from the control group that had been treated with 10% Suc and 0.05% DMSO. Treatment with each inhibitor was carried out more than three times, using three independent lines of transgenic plants. As shown in Figure 2, Suc-inducible expression of the β-Amy:GUS fusion gene was strongly inhibited by 0.5 μM okadaic acid, microcystin-LR, and calyculin A, and each of the inhibitors also had a reduced inhibitory effect at 0.05 μM.

Similar to the induction of accumulation of mRNAs for sporamin and β-amylase in leaf-petiole cuttings of sweet potato (Hattori et al., 1991; Nakamura et al., 1991), expression of the β-Amy:GUS fusion gene in leaves of transgenic tobacco can be induced by Glc or Fru instead of by Suc. Okadaic acid (0.5 μM) blocked the induction of the fusion gene by 0.3 M of Glc or Fru (Fig. 3), the results suggesting that the inhibition is not due to the inhibition of uptake and

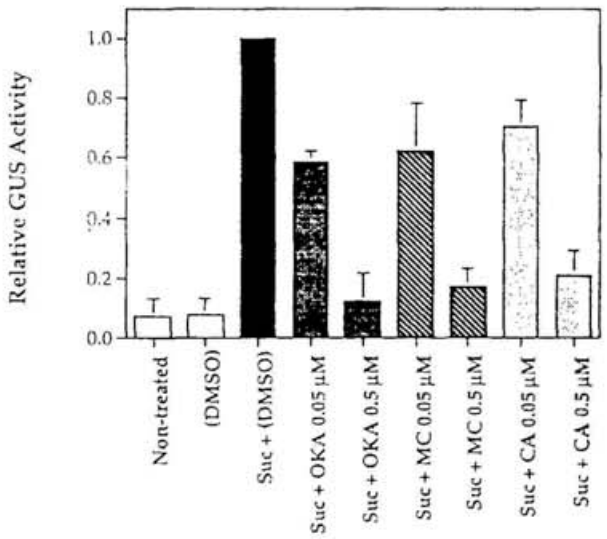


Figure 2. Effects of inhibitors of PP1 and PP2A on the Suc-inducible expression of the β-Amy:GUS fusion gene in leaf segments of transgenic tobacco. Leaf segments were treated with 10% Suc and 0.05% DMSO in the presence of 0.05 or 0.5 μM of okadaic acid (Suc + OKA), microcystin-LR (Suc + MC), and calyculin A (Suc + CA) or in their absence [Suc + (DMSO)], for 20 h. (DMSO) indicates leaf segments treated with 0.05% DMSO alone for 20 h, and Non-treated indicates nontreated leaf segments. Specific activities of GUS were normalized to that of control samples treated with Suc and DMSO, and they are presented as the means of results of independent experiments with SD (n = 3–12 for each treatment). Specific activities of GUS in leaf segments treated with Suc for 20 h varied between 435 and 21,000 pmol methylumbelliferone min^{-1} mg^{-1} protein, depending on the plants used.

metabolic cleavage of Suc. Mannitol and sorbitol did not induce the expression of the fusion gene (data not shown).

Figure 4 shows the time course of the increases in GUS activity in leaf segments after treatment with Suc in the presence or absence of 0.5 μM okadaic acid. The presence of okadaic acid at the onset of treatment with Suc almost completely blocked the Suc-induced increase in GUS activity. To examine whether the protein phosphatase activity is required only at the initial stage of induction by Suc, leaf segments that had been treated with Suc for 6 h were transferred to agar plates that contained 0.5 μM okadaic acid in addition to Suc. The GUS activity increased for the subsequent 6 h to a somewhat lower level than that found in leaf segments treated with Suc in the absence of okadaic acid, and further increases in GUS activity after that time were almost completely inhibited. These results suggest that the continuous dephosphorylation of protein(s) is required for the maintenance of sugar-induced expression of the fusion gene.

DISCUSSION

In this study, we used three structurally unrelated inhibitors of PP1 and PP2A, namely okadaic acid, microcystin-LR, and calyculin A, to examine the involvement of protein

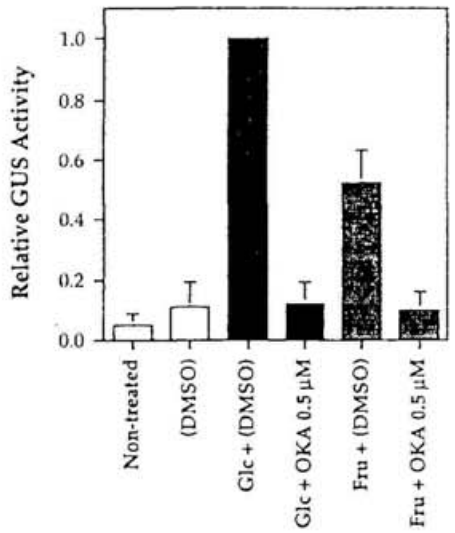


Figure 3. Effects of okadaic acid on the induction of expression of the β-Amy:GUS fusion gene in leaf segments of transgenic tobacco by Glc or Fru. Leaf segments were treated with 0.3 M Glc (Glc) or Fru (Fru) and 0.05% DMSO in the absence or the presence of 0.5 μM of okadaic acid (OKA) for 20 h. (DMSO) indicates leaf segments treated with 0.05% DMSO alone for 20 h, and Non-treated indicates nontreated leaf segments. Specific activities of GUS were normalized to that of control samples treated with Glc and DMSO, and they are presented as the means of results of independent experiments with SD ($n = 3$ for each treatment). Specific activities of GUS in leaf segments treated with Glc for 20 h varied between 9,500 and 11,300 pmol methylumbelliferone min^{-1} mg^{-1} protein, depending on the plants used.

dephosphorylation in the response of plant genes to carbohydrate metabolic signals. Expression of genes coding for two major proteins of tuberous roots of sweet potato, namely sporamin and β-amylase, was used as a model. Although the mRNAs for sporamin and β-amylase are usually not detectable in organs other than the tuberous root, they are inducible in stems, leaves, and petioles by exogenous supply of high concentrations of Suc or other metabolizable sugars (Hattori et al., 1990, 1991; Nakamura et al., 1991). All of the inhibitors of PP1 and PP2A examined strongly blocked the Suc-inducible accumulation of mRNAs for sporamin and β-amylase in leaf-petiole cuttings (Fig. 1; Table I). Concentrations of inhibitors of PP1 and PP2A used in our experiments were similar to those used in other in vivo experiments with plant tissues and cells (Siegl et al., 1990; Dominov et al., 1992; Raz and Fluhr, 1993; Sheen, 1993; MacKintosh et al., 1994). Among the mRNAs examined in this study, the inhibitory effects of phosphatase inhibitors were specific to mRNAs for sporamin, β-amylase, and the small subunit of AGPase (Fig. 1; Table I). These results suggest that the dephosphorylation of protein(s) is required for the Suc-inducible expression of genes for sporamin and β-amylase in sweet potato.

Accumulation of large amounts of starch occurs concomitantly with the accumulation of mRNAs for sporamin and β-amylase in leaf-petiole cuttings after treatment with metabolizable sugars. In addition, it is dependent on various sugars and on the concentration of Suc in ways that are similar to those of the accumulation of sporamin and β-amylase (Nakamura et al., 1991). Similar to the accumulation in tuberous roots, Suc-induced accumulation of sporamin, β-amylase, and starch occurs within the same cells in leaves and petioles. These results suggest that there is a close link among the accumulation of sporamin, β-amylase, and starch as part of the cellular expression of the vegetative storage function that is normally exhibited in parenchymatous cells of the tuberous root (Nakamura et al., 1991). In potato, sugar-inducible expression of class I patatin genes is accompanied by the accumulation of starch (Rocha-Sosa et al., 1989; Wenzler et al., 1989). Starch synthesis in plants is regulated at the level of AGPase (Preiss, 1988), and a potato gene for one of the two subunits of AGPase is inducible by metabolizable sugars (Müller-Röber et al., 1990).

In the present study, we observed a dramatic increase in the level of mRNA for the small subunit of AGPase after treatment of leaf-petiole cuttings with Suc for 12 h (Fig. 1), which occurred much earlier than the increase in the amount of starch (Nakamura et al., 1991). It seems that the accumulation of starch after exogenous supply of Suc is regulated at the level of gene expression, in addition to the increased level of substrates and the possible activation by 3-phosphoglyc-

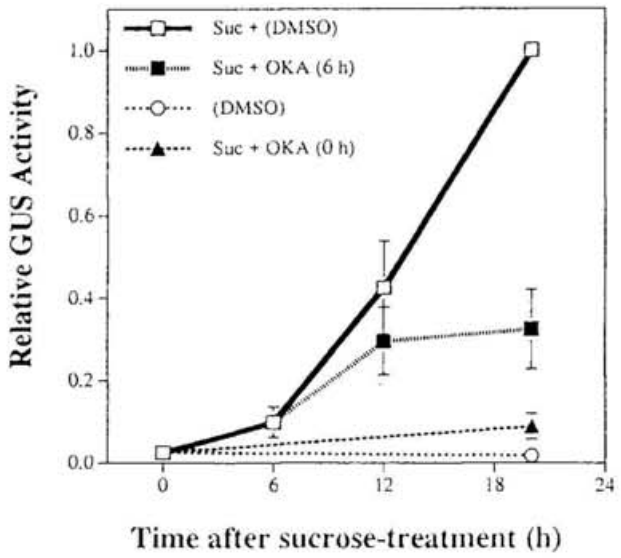


Figure 4. The time course of the increases in GUS activity in leaf segments of transgenic tobacco that carried the β-Amy:GUS fusion gene after treatment with Suc in the absence or presence of okadaic acid. Leaf segments were treated with 10% Suc and 0.05% DMSO in the absence [Suc + (DMSO)] or in the presence of 0.5 μM okadaic acid (OKA) at the same time as the treatment with Suc was begun (0 h) or 6 h after the start of treatment with Suc (6 h). (DMSO) indicates leaf segments treated with 0.05% DMSO alone. Specific activities of GUS were normalized to that from the control samples that had been treated with Suc and DMSO for 20 h. Results are presented as the means of results of five independent experiments with SD. Specific activities of GUS in leaf segments that had been treated with Suc and DMSO for 20 h varied between 2,200 and 37,000 pmol methylumbelliferone min^{-1} mg^{-1} protein, depending on the plants used.

erate (Preiss, 1988). The regulation of expression of genes coding for the large and small subunits of AGPase of sweet potato in relation to starch accumulation is currently under investigation in our laboratory. The Suc-induced accumulation of mRNA for the small subunit of AGPase was inhibited by inhibitors of PP1 and PP2A (Fig. 1), and its sensitivity to okadaic acid and to microcystin-LR was similar to that of the mRNAs for sporamin and β-amylase (Table I). These results raise the possibility that a similar protein phosphatase is involved in the regulation of expression of these genes, thereby connecting the regulation of the accumulation of starch with the accumulation of sporamin and β-amylase. We have not examined the effects of inhibitors of protein phosphatases on the accumulation of starch, since it required several days of treatment with Suc (Nakamura et al., 1991). Glycogen synthesis in mammals and yeasts is regulated by dephosphorylation and activation of glycogen synthase and dephosphorylation and inactivation of glycogen phosphorylase. A specific PP1 catalyzes these dephosphorylation reactions (Dent et al., 1990; François et al., 1992). In plants, PP1 and PP2A activities were not associated with chloroplasts in leaves of pea and wheat or starch granules in seeds of oilseed rape (MacKintosh et al., 1991). Protein dephosphorylation may be involved in the regulation of starch synthesis in plants at steps that are different from the regulation of glycogen synthesis in animals and yeasts.

Inhibitors used in this study can differentiate between PP1 and PP2A in in vitro experiments. Okadaic acid inhibits PP2A in the picomolar range, whereas it inhibits PP1 in the nanomolar range (Cohen, 1989; Cohen et al., 1990). Microcystin-LR and calyculin A inhibit PP1 and PP2A with almost equal potency, and they are more potent inhibitors of PP1 than is okadaic acid (Ishihara et al., 1989; MacKintosh et al., 1990). The effective concentration of okadaic acid in our experiments (0.02–0.2 μM; Table I) may suggest the involvement of PP1 in the Suc-inducible accumulation of mRNAs for sporamin and β-amylase. However, okadaic acid was more effective than microcystin-LR (Table I). The actual concentration of the inhibitor in the cell that could be affected by the permeability and stability of each inhibitor in our experimental conditions is not known. It is also not known whether one or more of PP1 or PP2A was involved. Thus, it is difficult to determine whether protein phosphatase(s) involved in the sugar-inducible gene expression in our experiments was PP1 or PP2A.

Suc synthase that is required for the metabolic cleavage of Suc to UDP-Glc and Fru plays an important role in sink tissues. Similar to the observation with single-leaf stem cuttings of potato (Salanoubat and Belliard, 1989), exogenous supply of Suc to the leaf-petiole cuttings of sweet potato induced increases in the level of Suc synthase mRNA (Fig. 1). In contrast to mRNAs for sporamin, β-amylase, and the small subunit of AGPase, the Suc-induced increase in the level of Suc synthase mRNA was significantly enhanced by 2 μM okadaic acid (Fig. 1; Table I). An enhanced level of Suc synthase mRNA was observed with a much higher concentration of okadaic acid than that required to inhibit the Suc-inducible accumulation of mRNAs for sporamin, β-amylase, and the small subunit of AGPase (Table I). Furthermore, the level of Suc synthase mRNA was not severely affected by microcystin-LR and calyculin A (Fig. 1; Table I). Although the protein phosphatase may also be involved in the Suc-inducible increase in the level of Suc synthase mRNA, it may be different from that involved in the sugar-inducible expression of the genes for sporamin, β-amylase, and the small subunit of AGPase.

Inhibition of Suc-inducible expression of the β-Amy:GUS fusion gene in leaf segments of transgenic tobacco by inhibitors of PP1 and PP2A (Fig. 2) suggests that the involvement of protein dephosphorylation in the sugar-inducible gene expression is conserved in different plant species and tissues. Effective concentrations of inhibitors in experiments with leaf segments of tobacco did not differ significantly from those observed in experiments with the petiole parts of leaf-petiole cuttings of sweet potato except that microcystin-LR was slightly more effective in leaf segments of tobacco (Table I; Fig. 2).

It is not clear which step(s) in the sugar-inducible gene expression is regulated by PP1 and/or PP2A. The specificity of the effects of inhibitors of PP1 and PP2A on different mRNAs (Fig. 1; Table I) indicates that these inhibitors do not affect the general transcriptional activity or the general stability of mRNAs under our conditions. Furthermore, the observations that okadaic acid does not inhibit the synthesis of the GUS polypeptide in maize protoplasts (Sheen, 1993) and induces the synthesis of pathogenesis-related proteins in tobacco (Raz and Fluhr, 1993) indicate that at least the effects of okadaic acid are not due to the general inhibition of protein synthesis. Okadaic acid not only blocked the Suc-induced expression of the β-Amy:GUS fusion gene in leaves of tobacco (Fig. 2) but also blocked the induction by Glc or Fru (Fig. 3). These results indicate that the inhibition is not due to the inhibition of the uptake and transport of Suc into the cell or the metabolic cleavage of Suc. It is suggested that protein dephosphorylation is involved in the transduction of carbohydrate metabolic signals to the nucleus.

PP1 and/or PP2A may regulate the activities of enzymes in the metabolism of sugars in the cell, thereby affecting the level of metabolic signal molecules themselves. It has been shown that PP2A regulates the activities of various plant enzymes, such as PEP carboxylase (Carter et al., 1990), quinate dehydrogenase (MacKintosh et al., 1991), Suc-phosphate synthase (Siegl et al., 1990; Huber and Huber, 1992), and nitrate reductase (Huber et al., 1992). Alternatively, direct protein dephosphorylation of factors involved in the transcriptional regulation of gene expression in the nucleus might be necessary. The results presented in Figure 4 suggest that PP1 and/or PP2A activity is required not only for the Suc-induced activation of gene expression but also for the maintenance of such expression. This possibility implies that continuous supply of phosphorylated substrate(s) is also required. The activity of the target protein of PP1 and/or PP2A may be modulated by reversible phosphorylation and dephosphorylation, and its phosphorylation status could be under the carbohydrate metabolic control.

Recently, the involvement of PP1 and/or PP2A activity in the response of plant genes to light (Sheen, 1993), phytohormones (Dominov et al., 1992; Raz and Fluhr, 1993), and fungal elicitors (MacKintosh et al., 1994) were reported. Okadaic acid itself induced pathogenesis-related proteins in

leaves of tobacco (Raz and Fluhr, 1993) and Phe ammonia-lyase in soybean cell culture (MacKintosh et al., 1994), and these inductions by okadaic acid were blocked by inhibitors of protein kinase. It is suggested that kinase and phosphatase are acting on the same protein and the phosphorylated form of the protein represents the active form (Raz and Fluhr, 1993). By contrast, light-inducible gene expression in maize protoplasts was blocked by okadaic acid and calyculin A, and it was suggested that PP1 activity is required as a positive regulator in the light-signaling pathways (Sheen, 1993). However, the specific protein phosphatase and its target protein that were involved in the signal transduction pathways were not identified in any of these cases.

PP1 and PP2A in animals are known to be composed of catalytic subunits and regulatory subunits (Cohen, 1989). In plants, catalytic subunits of PP1 and PP2A are encoded by a family of related genes (Smith and Walker, 1991, 1993; Ariño et al., 1993). Although catalytic subunits of plant PP1 and PP2A may also be associated with the other components (MacKintosh et al., 1991), little is known about the regulatory subunits of plant enzymes. In one of the most extensively studied cases in plants, a PP2A that dephosphorylates and activates Suc-phosphate synthase in spinach leaves is activated by light and inhibited by Pi (Weiner et al., 1992). To further clarify the role of protein dephosphorylation in the sugar-inducible gene expression, it is necessary to search for specific protein phosphatase and its target protein(s) that are involved in the pathways.

ACKNOWLEDGMENTS

We thank Mr. Tomiji Izuhara and Dr. Shigekata Yoshida of Nagoya University Experimental Farm for growing the sweet potato plants and Dr. Atsushi Kato of Hokkaido University and Dr. Hirofumi Uchimiya of Tokyo University for rice GAPDH cDNA.

Received May 2, 1994; accepted June 28, 1994.
Copyright Clearance Center: 0032-0889/94/106/0567/08.

LITERATURE CITED

Ariño J, Pérez-Callejón E, Cunillera N, Camps M, Posas F, Ferrer A (1993) Protein phosphatases in higher plants: multiplicity of type 2A phosphatases in *Arabidopsis thaliana*. Plant Mol Biol **21:** 475–485

Carter PJ, Nimmo HG, Fewson CA, Wilkins MB (1990) *Bryophyllum fedtschenkoi* protein phosphatase type 2A can dephosphorylate phosphoenolpyruvate carboxylase. FEBS Lett **263:** 233–236

Cheng C-L, Acedo GN, Cristinsin M, Conkling MA (1992) Sucrose mimics the light induction of *Arabidopsis* nitrate reductase gene transcription. Proc Natl Acad Sci USA **89:** 1861–1864

Cohen P (1989) The structure and regulation of protein phosphatases. Annu Rev Biochem **58:** 453–508

Cohen P, Holmes CFB, Tsukitani Y (1990) Okadaic acid: a new probe for the study of cellular regulation. Trends Biochem Sci **15:** 98–102

Dent P, Lavoinne A, Nakielny S, Caudwell FB, Watt P, Cohen P (1990) The molecular mechanism by which insulin stimulates glycogen synthesis in mammalian skeletal muscle. Nature **348:** 302–308

Dominov JA, Stenzler L, Lee S, Schwarz JJ, Leisner S, Howell SH (1992) Cytokinins and auxins control the expression of a gene in *Nicotiana plumbaginifolia* cells by feedback regulation. Plant Cell **4:** 451–461

François JM, Thompson-Jaeger S, Skroch J, Zellenka U, Spevak W, Tatchell K (1992) *GAC1* may encode a regulatory subunit for protein phosphatase type 1 in *Saccharomyces cerevisiae*. EMBO J **11:** 87–96

Hattori T, Fukumoto H, Nakagawa S, Nakamura K (1991) Sucrose-induced expression of genes coding for tuberous root storage protein, sporamin, of sweet potato in leaves and petioles. Plant Cell Physiol **32:** 79–86

Hattori T, Nakagawa T, Maeshima M, Nakamura K, Asahi T (1985) Molecular cloning and nucleotide sequence of cDNA for sporamin, the major soluble protein of sweet potato tuberous roots. Plant Mol Biol **5:** 313–320

Hattori T, Nakagawa S, Nakamura K (1990) High-level expression of tuberous root storage protein genes of sweet potato in stems of plantlets grown *in vitro* on sucrose medium. Plant Mol Biol **14:** 595–604

Huber JLA, Huber SC (1992) Site-specific serine phosphorylation of spinach leaf sucrose-phosphate synthase. Biochem J **283:** 877–882

Huber JL, Huber SC, Campbell WH, Redinbaugh MG (1992) Reversible light/dark modulation of spinach leaf nitrate reductase activity involves protein phosphorylation. Arch Biochem Biophys **296:** 58–65

Ishihara H, Martin BL, Brautigan DL, Karaki H, Ozaki H, Kato Y, Fusetani N, Watabe S, Hashimoto K, Uemura D, Hartshorne DJ (1989) Calyculin A and okadaic acid: inhibitors of protein phosphatase activity. Biochem Biophys Res Commun **159:** 871–877

Jefferson B-J, Kavanash TA, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J **6:** 3901–3907

Johnson R, Ryan CA (1990) Wound-inducible potato inhibitor II genes: enhancement of expression by sucrose. Plant Mol Biol **14:** 527–536

Koch KE, Nolte KD, Duke ER, McCarty DR, Avigne WT (1992) Sugar levels modulate differential expression of maize sucrose synthase genes. Plant Cell **4:** 59–69

MacKintosh C, Beattie KA, Klumpp S, Cohen P, Codd GA (1990) Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. FEBS Lett **264:** 187–192

MacKintosh C, Coggins J, Cohen P (1991) Plant protein phosphatases: subcellular distribution, detection of protein phosphatase 2C and identification of protein phosphatase 2A as the major quinate dehydrogenase phosphatase. Biochem J **273:** 733–738

MacKintosh C, Lyon GD, MacKintosh RW (1994) Protein phosphatase inhibitors activate anti-fungal defence responses of soybean cotyledons and cell cultures. Plant J **5:** 137–147

Müller-Rober BT, Koßmann J, Hannah LC, Willmitzer L, Sonnewald U (1990) One of two different ADP-glucose pyrophosphorylase genes from potato responds strongly to elevated levels of sucrose. Mol Gen Genet **224:** 136–146

Nakamura K, Ohto M, Yoshida N, Nakamura K (1991) Sucrose-induced accumulation of β-amylase occurs concomitant with the accumulation of starch and sporamin in leaf-petiole cuttings of sweet potato. Plant Physiol **96:** 902–909

Nakata PA, Greene TW, Anderson JM, Smith-White BJ, Okita TW, Preiss J (1991) Comparison of the primary sequences of two potato tuber ADP- glucose pyrophosphorylase subunits. Plant Mol Biol **17:** 1089–1093

Ohta S, Hattori T, Morikami A, Nakamura K (1991) High-level expression of a sweet potato sporamin gene promoter: β-glucuronidase (GUS) fusion gene in the stems of transgenic tobacco plants is conferred by multiple cell type-regulatory elements. Mol Gen Genet **225:** 369–378

Ohto M, Nakamura-Kito K, Nakamura K (1992) Induction of expression of genes coding for sporamin and β-amylase by polygalacturonic acid in leaf-petiole cuttings of sweet potato. Plant Physiol **99:** 422–427

Preiss J (1988) Biosynthesis of starch and its regulation. *In* J Preiss, ed, The Biochemistry of Plants, Vol 14. Academic Press, San Diego, CA, pp 181–254

Raz V, Fluhr R (1993) Ethylene signal is transduced via protein phosphorylation events in plants. Plant Cell **5:** 523–530

Rocha-Sosa M, Sonnewald U, Frommer W, Stratmann M, Schell

J, **Willmitzer L** (1989) Both developmental and metabolic signals activate the promoter of a class I patatin gene. EMBO J **8:** 23–29

Sakajo S, Nakamura K, Asahi T (1987) Molecular cloning and nucleotide sequence of full-length cDNA for sweet potato catalase mRNA. Eur J Biochem **165:** 437–442

Salanoubat M, Belliard G (1989) The steady-state level of potato sucrose synthase mRNA is dependent on wounding, anaerobiosis and sucrose concentration. Gene **84:** 181–185

Sheen J (1990) Metabolic repression of transcription in higher plants. Plant Cell **2:** 1027–1038

Sheen J (1993) Protein phosphatase activity is required for light-inducible gene expression in maize. EMBO J **12:** 3497–3505

Siegl G, MacKintosh C, Stitt M (1990) Sucrose-phosphate synthase is dephosphorylated by protein phosphatase 2A in spinach leaves. FEBS Lett **270:** 198–202

Smith RD, Walker JC (1991) Isolation and expression of a maize type 1 protein phosphatase. Plant Physiol **97:** 677–683

Smith RD, Walker JC (1993) Expression of multiple type 1 phosphoprotein phosphatases in *Arabidopsis thaliana*. Plant Mol Biol **21:** 307–316

Tsukaya H, Ohshima T, Naito S, Chino M, Komeda Y (1991) Sugar- dependent expression of *CHS-A* gene for chalcone synthase from petunia in transgenic *Arabidopsis*. Plant Physiol **97:** 1414–1421

Weiner H, McMichael RW Jr, Huber SC (1992) Identification of factors regulating the phosphorylation status of sucrose-phosphate synthase *in vivo*. Plant Physiol **99:** 1435–1442

Wenzler HC, Mignery GA, Fisher LM, Park WD (1989) Analysis of a chimeric class-I patatin-GUS gene in transgenic potato plants: high-level expression in tubers and sucrose-inducible expression in cultured leaf and stem explants. Plant Mol Biol **12:** 41–50

Yoshida N, Hayashi K, Nakamura K (1992) A nuclear gene encoding β- amylase of sweet potato. Gene **120:** 255–259

Yoshida N, Nakamura K (1991) Molecular cloning and expression in *Escherichia coli* of cDNA encoding the subunit of sweet potato β-amylase. J Biochem (Tokyo) **110:** 196–201

Yu S-M, Kuo Y-H, Sheu G, Sheu Y-J, Liu L-F (1991) Metabolic derepression of α-amylase gene expression in suspension-cultured cells of rice. J Biol Chem **266:** 21131–21137

Plant Cell Physiol. 37(7): 941–948 (1996)

cDNA Cloning and Expression of a Gene for Isocitrate Lyase in Pumpkin Cotyledons

Shoji Mano [1,2], Makoto Hayashi [1,2], Maki Kondo [1] and Mikio Nishimura [1,2,3]

[1] *Department of Cell Biology, National Institute for Basic Biology, Okazaki, 444 Japan*
[2] *Department of Molecular Biomechanics, School of Life Science, the Graduate University for Advanced Studies, Okazaki, 444 Japan*

A cDNA clone for isocitrate lyase (ICL) was isolated from a cDNA library prepared from the poly(A)$^+$ RNA of etiolated pumpkin cotyledons. The cDNA encoded a polypeptide with 576 amino acids, whose sequence is more than 79% identical to those of ICL from other higher plants and contains the C-terminal tripeptide, Ser-Arg-Met, which is a putative targeting signal to microbodies. Immunogold analysis revealed that ICL protein is exclusively localized in microbodies in etiolated pumpkin cotyledons. Double labeling experiments with protein A-gold particles of different sizes showed that ICL protein coexists with a leaf-peroxisomal enzyme, glycolate oxidase, in the microbodies of greening and senescing pumpkin cotyledons, indicating that transformation between glyoxysomes and leaf peroxisomes occurs directly during greening and senescence of the cotyledons. Immunoblot analysis showed that the amount of ICL protein increased markedly during germination and decreased rapidly when seedlings were exposed to illumination, which induced the microbody transition from glyoxysomes to leaf peroxisomes. In senescing cotyledons, the level of the ICL protein and ICL mRNA increased again with the reverse transition of microbodies from leaf peroxisomes to glyoxysomes. Changes in the amount of ICL protein did not correspond to the changes in the level of ICL mRNA during greening and senescence of the cotyledons, an indication that post-transcriptional regulation plays an important role in the microbody transition.

Key words: Glyoxysome — Isocitrate lyase (EC 4.1.3.1) — Leaf peroxisome — Microbody transition — Senescence.

Oilseed plants such as pumpkin and cucumber utilize large amounts of storage oil as the energy for seedling growth. Glyoxysomes, which are specialized microbodies in higher plants and contain enzymes for β-oxidation and the glyoxylate cycle, participate in the degradation of storage oil. During greening of the cotyledons in some oilseeds such as pumpkin and watermelon, glyoxysomes are transformed to another type of specialized microbody, namely, leaf peroxisomes, which function together with chloroplasts and mitochondria in photorespiration (Beevers 1979). In the microbody transition, activities of glyoxysomal enzymes such as malate synthase (MS; EC 4.1.3.2) and isocitrate lyase (ICL; EC 4.1.3.1) decreased, whereas activities of leaf-peroxisomal enzymes, such as glycolate oxidase (GO; EC 1.1.3.1) and hydroxypyruvate reductase (HPR; EC 1.1.1.29), increased. Our previous studies showed that during the microbody transition, the degradation of glyoxysomal enzymes was induced (Kato et al. 1995, Mori et al. 1991) and the amounts of mRNAs for leaf-peroxisomal enzymes markedly increased (Tsugeki et al. 1993). The reverse transition of leaf peroxisomes to glyoxysomes occurs during senescence (Nishimura et al. 1993). In this case, glyoxysomal enzymes are induced and leaf-peroxisomal enzymes are degraded. We have demonstrated immunocytochemically that this replacement of leaf-peroxisomal enzymes with glyoxysomal enzymes occurs in the same microbodies (Nishimura et al. 1993).

ICL is an enzyme unique to the glyoxylate cycle and catalyzes the cleavage of isocitrate into succinate and glyoxylate. cDNA for this enzyme has been characterized from cucumber (Reynolds and Smith 1995), rape (Comai et al. 1989), castor bean (Beeching and Northcote 1987), tomato (unpublished; accession U18678), cotton (Turley et al. 1990), glycine max (unpublished; accession LO2329), fungi (Barth and Scheuber 1993), yeast (Fernandez et al. 1992) and *E. coli* (Matsuoka and McFadden 1988). The synthesis and activity of this enzyme have been demonstrated in embryogenesis and in germinating seeds of higher plants (Allen et al. 1988, Comai et al. 1989, Turley et al. 1990, Weir et al. 1980, Zhang et al. 1993). A recent analysis showed that this enzyme was expressed in pollen from *Brassica napus* L. (Zhang et al. 1994) and was induced in detached or attached organs during senescence (De Bellis and Nishimura 1991, Graham et al. 1992, Gut and Matile 1988).

To obtain clues on the structural characteristics and the expression of pumpkin ICL during the reversible microbody transition, we cloned the cDNA for pumpkin ICL. We report here that the deduced amino acid sequence of pumpkin ICL contains Ser-Arg-Met at the C-terminal end and that it exclusively localizes in glyoxysomes. We also report the developmental changes in the levels of ICL pro-

Abbreviations: ICL, isocitrate lyase; MS, malate synthase; GO, glycolate oxidase; HPR, hydroxypyruvate reductase.

The nucleotide sequence reported in this paper has been submitted to the DDBJ, EMBL and GenBank nucleotide sequence databases under the accession number D78256.

[3] To whom corespondence should be addressed.

tein and ICL mRNA during the reversible microbody transition in pumpkin cotyledons.

Materials and Methods

Plant material—Pumpkin (*Cucurbita* sp. cv. Kurokawa Amakuri Nankin) seeds were soaked overnight and germinated in moist rock fiber (66R; Nitto Bouseki, Chiba, Japan) at 25°C in darkness. Some of these seedlings were transferred to continuous illumination at the 4–5 day stage. Senescing cotyledons were prepared as described previously (Nishimura et al. 1993).

Isolation and sequencing of a cDNA clone for pumpkin isocitrate lyase—Poly(A)$^+$ RNA was prepared from 4-day-old etiolated pumpkin cotyledons by SDS-phenol extraction with subsequent column chromatography on oligo (dT)-cellulose (Mori et al. 1991). An oligo (dT)-primed λgt11 cDNA library was constructed from the poly(A)$^+$ RNA using a cDNA synthesis system and a λgt11 cloning system (Amersham, Buckinghamshire, U.K.) according to the manufacturer's instructions. The recombinant phages were immunoscreened with an ICL-specific antibody, which had been kindly provided by Dr. M. Maeshima (Nagoya University, Nagoya, Japan). The cDNA insert from an immunopositive clone was subcloned into the plasmid vector pBluescript KS(+) (Stratagene, La Jolla, CA, U.S.A.) and used as a hybridization probe to identify longer cDNA clones from the same library by plaque hybridization. The longest cDNA insert obtained by plaque hybridization (1,983 bp), was subcloned into pBluescript KS(+). This plasmid was designated pICL11. Deletion mutants of the cDNA insert of pICL11 in both the sense and antisense strands were constructed using a deletion kit (Takara Shuzo, Kyoto, Japan). Nucleotide sequences of these deletion mutants were determined with an automatic DNA sequencer (model 373; Perkin Elmer/Applied Biosystems) according to the manufacturer's instructions. The nucleotide and the deduced amino acid sequences were analyzed with DNA analytical software (GeneWorks; IntelliGenetics, Mountain View, CA, U.S.A).

Northern blot analysis—Ten μg of total RNA extracted from pumpkin cotyledons grown under various conditions was fractionated on a 1% gel that contained 0.66 M formaldehyde and 10 mM MOPS (pH 7.0). RNA was transferred to a Hybond N$^+$ membrane (Amersham Japan) in 50 mM NaOH and fixed by exposure to UV-light (Funa-UV-Linker, model FS-800; Funakoshi, Tokyo, Japan). The insert corresponding to the isocitrate lyase cDNA was labeled with [^{32}P]dCTP (Amersham Japan). Ten μg of total RNA extracted from pumpkin cotyledons grown under various conditions was fractionated on a 1% agarose gel using a *Bca*Best labeling kit (Takara Shuzo, Kyoto, Japan). The membrane was hybridized in 0.5 M sodium phosphate (pH 7.2), 1 mM EDTA (pH 8.0), 7% SDS and 1% bovine serum albumin with 1.0×10^6 cpm ml^{-1} of radiolabeled DNA probe for 18 h at 42°C. The membrane was washed at 42°C in 2×SSC plus 0.1% SDS for 15 min, in 0.2× SSC plus 0.1% SDS for 15 min, in 0.1×SSC plus 0.1% SDS for 15 min and at 60°C twice for 15 min each. The membrane was exposed to X-ray film and radioactivity was measured on the imaging plate of a Bio-Imaging analyzer (FUJIX BAS2000; FUJI Photo Film, Tokyo) with an exposure time of 18 h.

Slot blot analysis—Changes in the level of ICL mRNA during senescence in vitro was examined by slot blot hybridization using DIG labeling and detection kits (Boehringer Mannheim, Tokyo, Japan). Total RNA was extracted from ten cotyledon halves at various stages. An aliquot of 0.33% of the total RNA was blotted onto a charged nylon membrane (Boehringer Mannheim, Tokyo, Japan). Antisense RNA was synthesized from pICL11 by T7 RNA polymerase. The antisense RNA, labeled with digoxygenin-conjugated UTP, was used as a probe for hybridization. Hybridization and washing conditions were those prescribed by the manufacturer and mRNA-probe hybrids were detected by monitoring the activity of alkaline phosphatase that had been conjugated to digoxygenin-specific antibodies. The intensity of each signal was quantitated with a densitometer (Densitograph, ver 2.0; ATTO).

Immunoblot analysis—Pumpkin cotyledons grown under various conditions were homogenized with SDS-loading buffer (100 mM Tris-HCl, pH 6.8, 1 mM EDTA, pH 8.0, 0.1% Triton X-100 and 1 mM PMSF) and then the homogenates were centrifuged at 15,000×*g* for 25 min. The protein content of each extract was estimated using a protein assay kit (Nippon Bio-Rad Laboratories, Tokyo, Japan) with bovine gamma albumin as a standard. Ten μg of total protein was separated by SDS-PAGE on a 12.5% polyacrylamide gel as described by Laemmli (1970) and transferred to a nylon membrane (Schleicher & Schuell, Dassel, Germany) in a semidry electroblotting system. The polypeptides on the membrane were visualized with 1 : 5,000 dilution of horseradish peroxidase antibodies against rabbit IgG (ECL system; Amersham, Japan).

Immunoelectron microscopy—Germinating pumpkin cotyledons were harvested at various stages and senescing cotyledons were harvested at 9 days after treatment. The samples were fixed, dehydrated and embedded in LR white resin (London Resin, U.K.) as described previously (Nishimura et al. 1993). Ultrathin sections were cut on a Reichert ultramicrotome (Leica, Heidelberg, Germany) with a diamond knife and mounted on uncoated nickel grids. The protein A-gold labeling procedure was essentially the same as that described by Nishimura et al. (1993). Ultra-thin sections were incubated at room temperature for 1 h with a solution of antiserum against ICL or glycolate oxidase (GO) diluted 1 : 100 and then with a 50-fold diluted suspension of protein A-gold (15 nm for GO, 10 nm for ICL; Amersham Japan) at room temperature for 30 min. The sections were examined with a transmission electron microscope (1200EX; JOEL, Tokyo, Japan) at 80 kV.

Results and Discussion

Cloning of a cDNA for isocitrate lyase—A λgt11 cDNA library from the poly(A)$^+$ RNA of 4-day-old etiolated pumpkin cotyledons was constructed. We screened this cDNA library immunologically using an antibody against castor bean ICL. Ten immunopositive clones were isolated from 1.5×10^6 independent recombinants. The same cDNA library was screened by plaque hybridization using the 1.4 kb insert of one of the immunopositive clones as a probe. We obtained eleven positive clones, including one that contained the longest insert of 1.9 kb. The complete nucleotide sequence and the deduced amino acid sequence are shown in Fig. 1. This cDNA consists of 1,983 bp and contains a 1,728-bp open reading frame that encodes a polypeptide of 576 amino acids with a total molecular mass of 64,286 Da. The nucleotide and deduced amino acid sequences of ICL have been reported for cucumber (Reynolds and Smith 1995), rape (Comai et al. 1989), castor bean (Beeching and Northcote 1987), tomato (unpublished; accession U18678), cotton (Turley et al. 1990), glycine

max (unpublished; accession LO2329), fungi (Barth and Scheuber 1993), yeast (Fernandez et al. 1992) and *E. coli* (Matsuoka and McFadden 1988). The identity with ICL amino acid sequences of other plants is 97% for cucumber, 85% for rape, 88% for castor bean, 85% for tomato, 89% for cotton and 79% for glycine max. It has been proposed that Leu-169, Lys-170, Pro-171, Thr-210, Lys-211 and Lys-212 in ICL from castor bean might be involved in substrate binding (Beeching and Northcote 1987). These amino acid residues are conserved in the corresponding positions of the pumpkin enzyme.

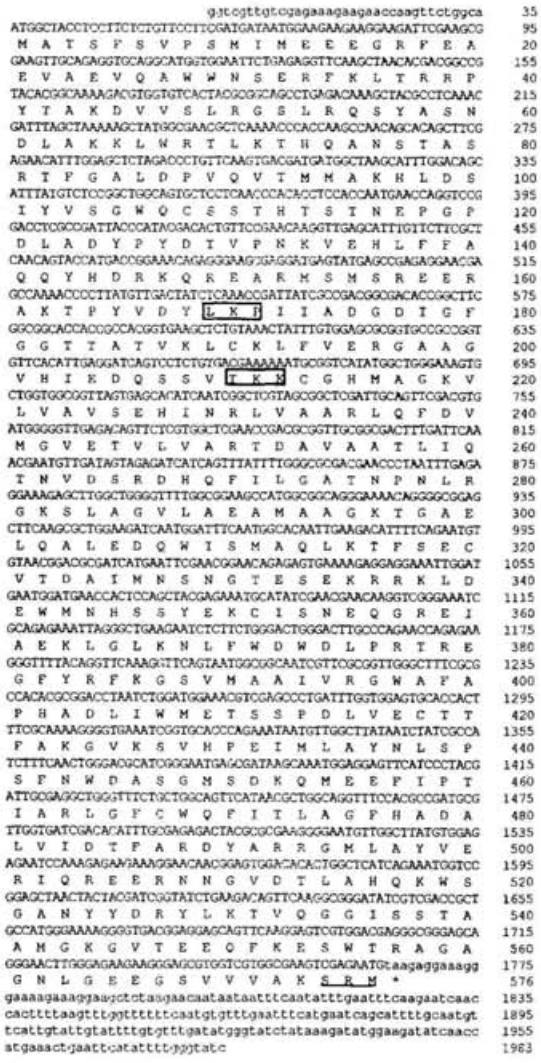


Fig. 1 Nucleotide and deduced amino acid sequences of pumpkin ICL cDNA. The nucleotide sequence of the putative coding region is shown in uppercase letters and the 5′ and 3′-noncoding regions are shown in lowercase letters. The deduced amino acid sequence is presented in the single-letter code beneath the nucleotide sequence, starting at the first in-frame methionine residue and ending at the first stop codon, indicated by an asterisk. The amino acid sequences tentatively involved in substrate-binding are boxed and the three carboxyl-terminal amino acids that are known as a microbody-targeting signal are underlined.

A putative signal for targeting of isocitrate lyase to microbodies—It has been proposed that a targeting signal, Ser-Lys-Leu or similar variants, is located at the C-terminal ends of various microbody proteins (Gould et al. 1988, 1989, Miyazawa et al. 1989). In fact, several kinds of specific C-terminal tripeptides have been found in glyoxysomal enzymes such as malate synthase (Graham et al. 1989, Mori and Nishimura 1989) as well as in leaf-peroxisomal enzymes such as glycolate oxidase (Tsugeki et al. 1993, Volokita and Somerville 1987) and hydroxypyruvate reductase (Hayashi et al. 1996b). Moreover, it has been demonstrated that the same protein targeting system is also functional in three different microbodies of higher plants, namely, glyoxysomes, leaf peroxisomes and non-specialized microbodies (Hayashi et al. 1996a). The C-terminal sequence of pumpkin ICL, like those of cucumber and rape, was Ser-Arg-Met while those of castor bean, tomato, cotton and glycine max were Ala-Arg-Met (Fig. 2). The latter tripeptide was shown to function as a targeting signal to microbodies (Trelease et al. 1994). As to the former, Ser-Arg-Met, Olsen et al. (1993) reported that this tripeptide was necessary for the transport of glyoxysomal proteins to microbodies in mammalian cells. Moreover, we recently obtained evidence using an in vivo targeting assay that employs transformants of the bacterial enzyme β-glucuronidase in which the carboxyl terminus is modified to Ser-Arg-Met, that the fusion protein was imported into microbodies (Hayashi et al. 1996a). It should be mentioned that the *E. coli* ICL lacks this C-terminal tripeptide (Fig. 2), since isocitrate lyase does not need to be subcompartmentalized in *E. coli*.

Developmental changes in the levels of isocitrate lyase after germination—We investigated developmental changes in the levels of ICL protein in pumpkin cotyledons after germination. Fig. 3 shows the changes in the accumulation of ICL protein. The relative amounts of ICL protein were determined densitometrically after immunoblotting. When seedlings were grown in darkness, the levels of ICL protein reached a maximum after seven days and then gradually decreased (closed circles in Fig. 3). However, the amount of ICL protein decreased rapidly (open circles in Fig. 3) when 5-day-old seedlings were transferred to illumination. In the case of other glyoxysomal enzymes, such as malate synthase and citrate synthase, similar developmental changes in their levels in pumpkin cotyledons have been observed during the transition of microbodies from glyoxysomes to leaf peroxisomes (Kato et al. 1995). These results suggest that the amount of pumpkin ICL is develop-

Pumpkin	530	KTVQGGISSTAAMGKGVTEEQFKESWTRAGAGNLGEEGSVVVAKSRM
Cucumber	530	KTVQGGISSTAAMGKGVTEEQFKESWTREGAVNLGEEGNVVVAKSRM
Rape	530	KTVQGGISSTAAMGKGVTEEQFKETWTRPGAAGNGEGTSLVVAKSRM
Castor bean	530	KTVQGGISSTAAMGKGVTEEQFKETWTRPGAMEMGSAGSEVVAKARM
Tomato	530	RTVQGGITSTAAMGKGVTEEQFEEKWTRTGATNLG-DGSVVIAKARM
Cotton	530	KTVQGGISSTAAMGKGVTEEQFKETWTRPGAGNIGSEGNLVVAKARM
Glycine max	511	KTVQGGVASTAAMGKGVTEEQFKESWTRPGAVEID-RGSIVVAKARM
Y. lipolytica	530	RMVTGGITSTAAMGAGVTEDQFKSKL----------
S. cerevisiae	529	KLAQGGVSATAAMGTGVTEDQFKENGVKK----------
E. coli	413	TIIQGG----------DVFSHRADRLH----------

Fig. 2 Alignment of C-terminal sequences of ICL polypeptide from different organisms. Sequences were taken from the references cited in the text. Amino acids are represented by standard one-letter codes. Residues that are conserved with respect to pumpkin ICL are highlighted.

mentally regulated, as are the amounts of other glyoxysomal enzymes.

Developmental changes in the levels of mRNA for isocitrate lyase—The changes in mRNA for ICL in various developmental stages were investigated by Northern analysis, as shown in Fig. 4. The levels of ICL mRNA appeared

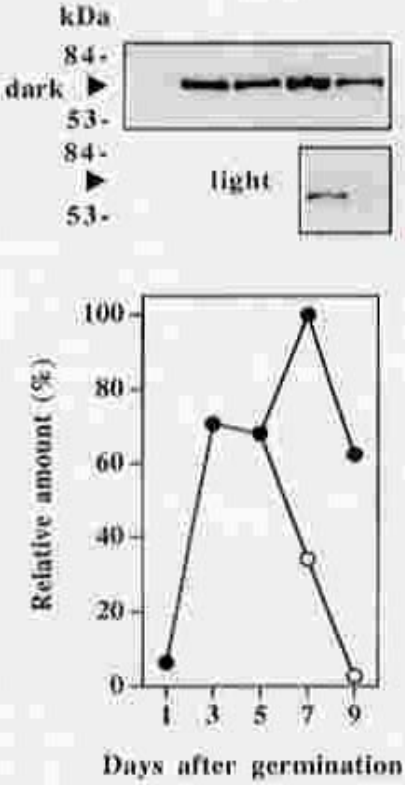


Fig. 3 Developmental changes in the relative amounts of ICL polypeptide in pumpkin cotyledons after germination. Total protein prepared from cotyledons grown under various conditions was subjected to SDS-PAGE (10 μg each), which was followed by immunodetection with an antibody against ICL. The ICL polypeptides are indicated by arrowheads. The relative amounts of the ICL polypeptides were determined densitometrically. (●) Seedlings grown in darkness; (○) seedlings exposed to continuous illumination at the 5-day stage.

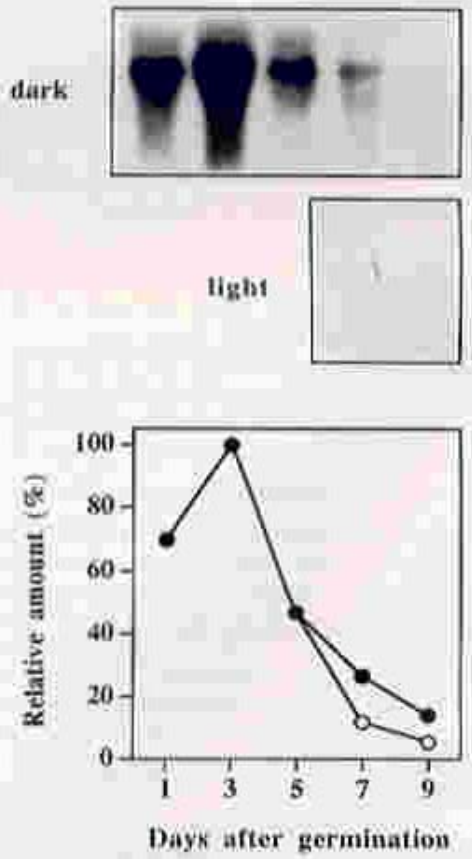


Fig. 4 Developmental changes in levels of ICL mRNA in pumpkin cotyledons after germination. Ten μg of total RNA extracted from pumpkin cotyledons grown under various conditions was separated on a 1% agarose gel. The RNA transferred to a Hybond N$^+$ membrane (Amersham) was hybridized with a [^{32}P]-labeled cDNA fragment for pumpkin ICL. Radioactivity was measured densitometrically. (●) Seedlings grown in darkness; (○) seedlings exposed to continuous illumination at the 5-day stage.

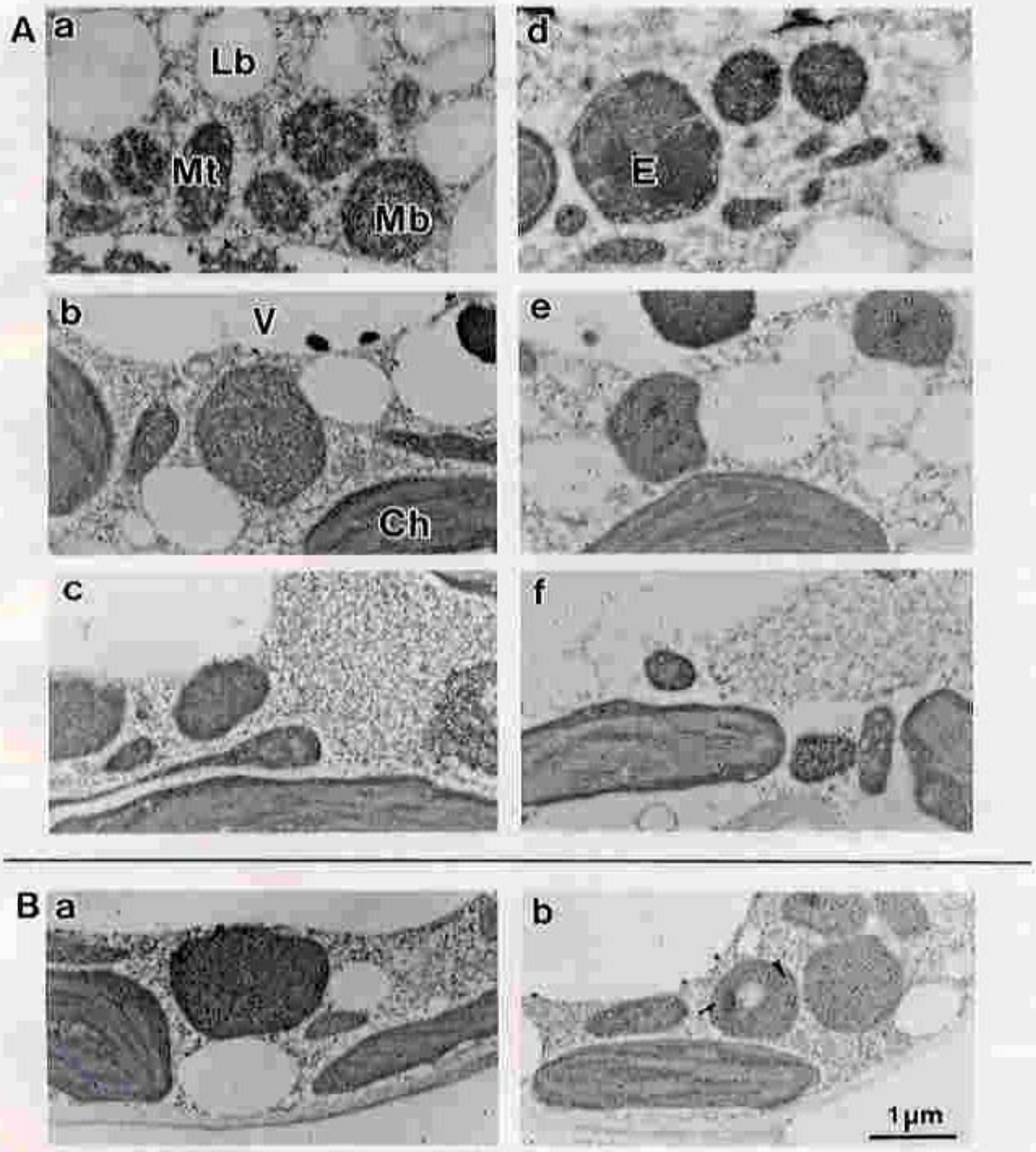


Fig. 5 Immunocytochemical localization of ICL in pumpkin cotyledons. Immunogold labeling of ultra-thin sections of pumpkin cotyledons was carried out using an antibody against ICL (A-a, b, c and B) and an antibody against a leaf-peroxisomal enzyme, glycolate oxidase (GO; A-d, e, f and B). (A) Electron micrograph of germinating pumpkin cotyledons. (a) and (d) 4-day dark-grown cotyledons, (b) and (e) 4-day dark- and 2-day light-grown cotyledons, (c) and (f) 4-day dark- and 4-day light-grown cotyledons. (B) Double immunogold labeling of 4-day dark- and 2-day light-grown cotyledons (a) and senescing cotyledons placed in darkness for 9 days after grown in green house for 20 days (b). Large gold particles (size 15 nm, arrowhead) label GO, and small gold particles (size 10 nm, arrow) label ICL. Symbols are the same as in panel A. Mb, microbody; Mt, mitochondrion; E, etioplast; Ch, chloroplast; V, vacuole; Lb, lipid body. Bar indicates 1 μm and refers to all samples.

to be high even in 1-day-old cotyledons, reached the maximum level for 3 days after germination and then declined to a low level. When cotyledons were exposed to continuous light, a slight decrease in the level of mRNA was observed (open circles in Fig. 4). However, the difference in mRNA levels between cotyledons grown in darkness and those exposed to light at the 5-day-old stage was not as remarkable as the difference observed in protein levels (Fig. 3). After 3 days, the amount of ICL mRNA dropped rapidly but the amount of ICL protein did not decrease. These results indicate that the amount of ICL protein in cotyledons of germinating pumpkin seeds does not correspond with the amount of transcript encoding this enzymes. In Southern blot analysis, the ICL probe hybridized to a single band of pumpkin genomic DNA that had been digested with *Spe*I and *Sma*I, which do not cut the ICL cDNA (data not shown). Since the copy number of the gene for ICL in pumpkin seems to be a single-copy per haploid genome, these results suggest that the amount of ICL protein is regulated post-transcriptionally. Similar results have been previously reported for malate synthase (Smith and Leaver 1986) and citrate synthase (Kato et al. 1995). Expression of genes coding for these glyoxysomal enzymes may be regulated similarly during the microbody transition.

Immunoelectron-microscope analysis of the localization of isocitrate lyase after germination—We investigated the localization of ICL and the changes in the amount of ICL protein after germination using immunoelectron microscopy. Gold particles for ICL were detected exclusively in microbodies but not in other organelles such as mitochondria, etioplasts and lipid bodies. The number of particles in microbodies decreased rapidly when continuous light was applied to seedlings at the 4-day stage (Fig. 5A; a, b, c). The change in the labeling density reflects the change in the level of ICL proteins. By contrast, gold particles specific for a leaf-peroxisomal enzyme, glycolate oxidase (GO), appeared and increased dramatically as a result of illumination (Fig. 5A; d, e, f). Tsugeki et al. (1993) reported that the expression of the gene for GO was induced dramatically by illumination. These electron micrographs clearly show that the microbody transition from glyoxysomes to leaf peroxisomes occurred during the greening of pumpkin cotyledons as described by Nishimura et al. (1993). If the microbody transition is caused by the replacement of glyoxysomal enzymes with leaf-peroxisomal enzymes, glyoxysomal and leaf-peroxisomal enzymes coexist within each microbody during the transition period. Fig. 5B-(a) shows the result of a double immunogold-labeling experiment in which cotyledons were grown in darkness for 4 days and exposed to light for 2 days and then sections were stained with protein A-gold particles of different sizes. It is clear that ICL (small particles) and GO (large particles) coexist in the same microbodies during the transition period, indicating that glyoxysomes are transformed directly to leaf peroxisomes during the microbody transition as reported by Nishimura et al. (1986) and Titus and Becker (1985).

Changes in the levels of isocitrate lyase during senescence in vitro—After greening, cotyledons gradually undergo senescence. Gut and Matile (1988) have shown that the glyoxysomal enzymes are detectable in detached senescent leaves. The appearance of glyoxysomal enzymes during senescence has been shown to be a reverse transition of leaf peroxisomes to glyoxysomes (De Bellis and Nishimura 1991). We investigated the changes in levels of ICL protein and ICL mRNA during senescence in vitro.

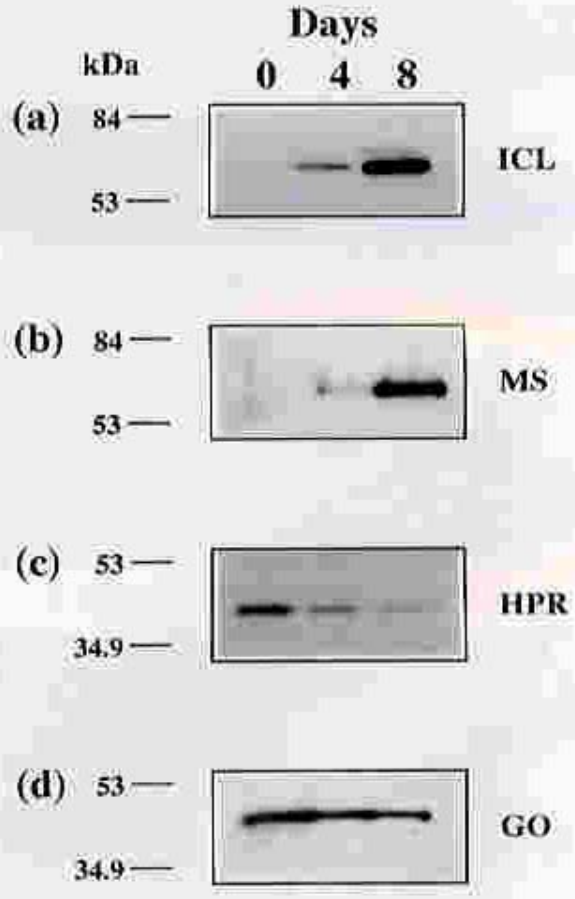


Fig. 6 Changes in levels of microbody-specific enzymes during senescence in vitro. Green cotyledons grown in a greenhouse for 20 days were placed in a plastic case in darkness. Total protein from cotyledons grown in darkness for 0, 4 and 8 days was subjected to SDS-PAGE (10 μg each), which was followed by immunodetection with an antibody against two glyoxysomal enzymes, ICL (panel a) and malate synthase (MS; panel b) and two leaf-peroxisomal enzymes, hydroxypyruvate reductase (HPR; panel c) and glycolate oxidase (GO; panel d). Molecular markers are indicated in kDa on the left.

After incubation of 20-day-old cotyledons in permanent darkness, the amount of ICL protein increased markedly (Fig. 6A), as did another glyoxysomal enzyme, malate synthase (MS) (Fig. 6B), whereas the leaf-peroxisomal enzymes, hydroxypyruvate reductase (HPR) and glycolate oxidase (GO), gradually decreased during senescence (Fig. 6C, D). We also detected the change at level of mRNA for ICL and MS (Fig. 7). The amount of ICL mRNA as well as that of MS mRNA increased as parallel during senescence. However, the change in levels of ICL protein did not correspond to those of the mRNAs. The accumulation of mRNA for ICL preceded with that of the protein during senescence as same as during germination. This developmental analysis of ICL during senescence suggests that post-transcriptional regulation also plays a role in the reverse transition of microbody. Although the function of ICL during senescence is still obscure, it is supposed that ICL may participate in the degradation of membranous lipids in the cells during senescence as a member of glyoxylate cycle.

In order to clarify the manner of the reverse transition, we performed an immunogold analysis with antibodies against ICL and GO. Fig. 5B-(b) clearly shows that ICL (*small* particles) and glycolate oxidase (large particles) coexist on the same microbodies in senescing cotyledons, indicating that leaf peroxisomes are directly transformed to glyoxysomes in senescing cotyledons. The replacement of leaf-peroxisomal enzymes with glyoxysomal enzymes was also observed in other organs such as petals (De Bellis et al. 1991). Moreover, these phenomena have been observed in naturally senescing leaves (De Bellis and Nishimura 1991, Pistelli et al. 1991). These results suggest that the microbody transition seems to be a reversible process between glyoxysomes and leaf peroxisomes. Further studies on the regulation of gene expression and degradation of glyoxysomal and leaf-peroxisomal enzymes will help to explain the reversibility of the microbody transition at the molecular level.

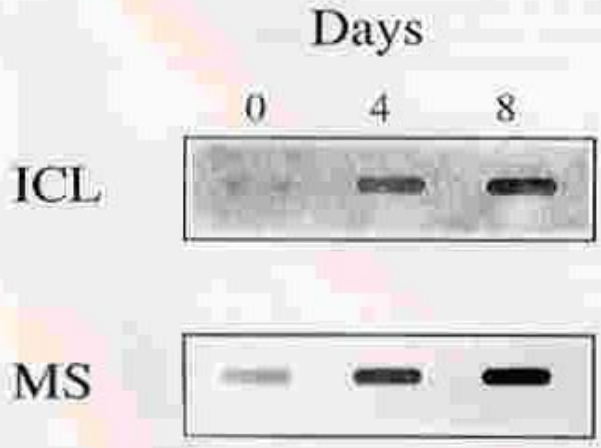


Fig. 7 Changes in levels of mRNAs for ICL and MS during senescence in vitro. An aliquot of 0.33% of the total RNA from ten cotyledon halves, which were treated as shown Figure 3, was blotted on a nylon membrane. Then the membrane was allowed to hybridize with the DIG-labeled RNA probe for ICL and MS.

The authors are most grateful to Dr. Masayoshi Maeshima (Nagoya University) for kindly providing the antibody against castor bean ICL. This work was supported by Grants-in-Aid for Scientific Research (B) (No. 07456053) and on Priority Areas (No. 04273101, 04273102) from the Ministry of Education, Science and Culture, Japan, by a grant from the Nissan Science Foundation (Tokyo, Japan) and by a grant from the DAIKO Foundation (Nagoya, Japan).

References

Allen, R.D., Trelease, R.N. and Thomas, T.L. (1988) Regulation of isocitrate lyase gene expression in sunflower. *Plant Physiol.* 86: 527–532.

Barth, G. and Scheuber, T. (1993) Cloning of the isocitrate lyase gene (*ICL1*) from *Yarrowia lipolytica* and characterization of the deduced protein. *Mol. Gen. Genet.* 241: 422–430.

Beeching, J.R. and Northcote, D.H. (1987) Nucleic acid (cDNA) and amino acid sequences of isocitrate lyase from castor bean. *Plant Mol. Biol.* 8: 471–475.

Beevers, H. (1979) Microbodies in higher plants. *Annu. Rev. Plant Physiol.* 30: 159–193.

Comai, L., Dietrich, R.A., Maslyar, D.J., Baden, C.S. and Harada, J.J. (1989) Coordinate expression of transcriptionally regulated isocitrate lyase and malate synthase genes in *Brassica napus* L. *Plant Cell* 1: 293–300.

De Bellis, L. and Nishimura, M. (1991) Development of enzymes of the glyoxylate cycle during senescence of pumpkin cotyledons. *Plant Cell Physiol.* 32: 555–561.

De Bellis, L., Tsugeki, R. and Nishimura, M. (1991) Glyoxylate cycle enzymes in peroxisomes isolated from petals of pumpkin (*Cucurbita* sp.) during senescence. *Plant Cell Physiol.* 32: 1227–1235.

Fernandez, E., Moreno, F. and Rodicio, R. (1992) The *ICL1* gene from *saccharomyces cerevisiae*. *Eur. J. Biochem.* 983–990.

Gould, S.J., Keller, G.-A., Hosken, N., Wilkinson, J. and Subramani, S. (1989) A conserved tripeptide sorts proteins to peroxisomes. *J. Cell Biol.* 108: 1657–1664.

Gould, S.J., Keller, G.-A. and Subramani, S. (1988) Identification of peroxisomal targeting signals located at the carboxy terminus of four peroxisomal proteins. *J. Cell Biol.* 107: 897–905.

Graham, I.A., Leaver, C.J. and Smith, S.M. (1992) Induction of malate synthase gene expression in senescent and detached organs of cucumber. *Plant Cell* 4: 349–357.

Graham, I.A., Smith, L.M., Brown, J.W.S., Leaver, C.J. and Smith, S.M. (1989) The malate synthase gene of cucumber. *Plant Mol. Biol.* 13: 673–684.

Gut, H. and Matile, P. (1988) Apparent induction of key enzymes of the glyoxylic acid cycle in senescent barley leaves. *Planta* 176: 548–550.

Hayashi, M., Aoki, M., Kato, A., Kondo, M. and Nishimura, M. (1996a) Transport of chimeric proteins that contain a carboxy-terminal targeting signal into plant microbodies. *Plant J.* 10: 225–234.

Hayashi, M., Tsugeki, R., Kondo, M., Mori, H. and Nishimura, M. (1996b) Pumpkin hydroxypyruvate reductases with and without a putative C-terminal signal for targeting to microbodies may be produced by alternative splicing. *Plant Mol. Biol.* 30: 183–189.

Kato, A., Hayashi, M., Mori, H. and Nishimura, M. (1995) Molecular characterization of a glyoxysomal citrate synthase that is synthesized as a precursor of higher molecular mass in pumpkin. *Plant Mol. Biol.* 27: 377–390.

Lacomb, O.K. (1970) Cleavage of structural proteins during assembly of

head of bacteriophage T4. *Nature* 227: 680–685.
Matsuoka, M. and McFadden, B.A. (1988) Isolation, hyperexpression, and sequencing of the *ace*A gene encoding isocitrate lyase in *Escherichia coli*. *J. Bacteriol.* 179: 4528–4536.
Miyazawa, S., Osumi, T., Hashimoto, T., Ohno, K., Miura, S. and Fujiki, Y. (1989) Peroxisome targeting signal of rat liver acyl-coenzyme A oxidase resides at the carboxy terminus. *Mol. Cell. Biol.* 9: 83–91.
Mori, H. and Nishimura, M. (1989) Glyoxysomal malate synthase is specifically degraded in microbodies during greening of pumpkin cotyledons. *FEBS Lett.* 244: 163–166.
Mori, H., Takeda-Yoshikawa, Y., Hara-Nishimura, I. and Nishimura, M. (1991) Pumpkin malate synthase. Cloning and sequence of the cDNA and Northern blot analysis. *Eur. J. Biochem.* 197: 331–336.
Nishimura, M., Takeuchi, Y., De Bellis, L. and Hara-Nishimura, I. (1993) Leaf peroxisomes are directly transformed to glyoxysomes during senescence of pumpkin cotyledons. *Protoplasma* 175: 131–137.
Nishimura, M., Yamaguchi, J., Mori, H., Akazawa, T. and Yokota, S. (1986) Immunocytochemical analysis shows that glyoxysomes are directly transformed to leaf peroxisomes during greening of pumpkin cotyledon. *Plant Physiol.* 80: 313–316.
Olsen, L.J., Ettinger, W.F., Damsz, B., Matsudaira, K., Webb, M.A. and Harada, J.J. (1993) Targeting of glyoxysomal proteins to peroxisomes in leaves and roots of a higher plant. *Plant Cell* 5: 941–952.
Pistelli, L., Bellis, L.D. and Alpi, A. (1991) Peroxisomal enzyme activities in attached senescing leaves. *Planta* 184: 151–153.
Reynolds, S.J. and Smith, S.M. (1995) The isocitrate lyase gene of cucumber: isolation, characterization and expression in cotyledons following seed germination. *Plant Mol. Biol.* 27: 487–497.
Smith, S.M. and Leaver, C.J. (1986) Glyoxysomal malate synthase of cucumber: molecular cloning of a cDNA and regulation of enzyme synthesis during germination. *Plant Physiol.* 81: 762–767.
Titus, D.E. and Becker, W.M. (1985) Investigation of the glyoxysome-peroxisome transition in germinating cucumber cotyledons using double-label immunoelectron microscopy. *J. Cell Biol.* 101: 1288–1299.
Trelease, R.N., Choe, S.M. and Jacobs, B.L. (1994) Conservative amino acid substitutions of the C-terminal tripeptide (Ala-Arg-Met) on cottoned isocitrate lyase preserve import in vivo into mammalian cell peroxisomes. *Eur. J. Cell Biol.* 65: 269–279.
Tsugeki, R., Hara-Nishimura, I., Mori, H. and Nishimura, M. (1993) Cloning and sequencing of cDNA for glycolate oxidase from pumpkin cotyledons and Northern blot analysis. *Plant Cell Physiol.* 34: 51–57.
Turley, R.B., Choe, S.M. and Trelease, R.N. (1990) Characterization of a cDNA clone encoding the complete amino acid sequence of cotton isocitrate lyase. *Biochim. Biophys. Acta* 1049: 223–226.
Volokita, M. and Somerville, C.R. (1987) The primary structure of spinach glycolate oxidase deduced from the DNA sequence of a cDNA clone. *J. Biol. Chem.* 262: 15825–15828.
Weir, E.M., Riezman, H., Grienenberger, J-M., Becker, W.M. and Leaver, C.J. (1980) Regulation of glyoxysomal enzymes during germination of cucumber: temporal changes in translatable mRNAs for isocitrate lyase and malate synthase. *Eur. J. Biochem.* 112: 469–477.
Zhang, J.Z., Gomez-Pedrozo, M., Baden, C.S. and Harada, J.J. (1993) Two classes of isocitrate lyase genes are expressed during late embryogeny and postgermination in *Brassica napus* L. *Mol. Gen. Genet.* 238: 177–184.
Zhang, J.Z., Laudencia-Chingcuanco, D.L., Comai, L., Li, M. and Harada, J.J. (1994) Isocitrate lyase and malate synthase genes from *Brassica napus* L. are active in pollen. *Plant Physiol.* 104: 857–864.

(Received May 7, 1996; Accepted July 19, 1996)

CELL STRUCTURE AND FUNCTION 21: 387–393 (1996)

Functional Transformation of Microbodies in Higher Plant Cells

Mikio Nishimura[1], Makoto Hayashi, Akira Kato, Katsushi Yamaguchi, and Shoji Mano

Department of Cell Biology, National Institute for Basic Biology, Okazaki 444, Japan

ABSTRACT. **In germinating fatty seedlings, microbodies are functionally transformed to leaf peroxisomes from glyoxysomes during greening, and then converted to glyoxysomes from leaf peroxisomes during senescence. Immunocytochemical studies revealed that glyoxysomes can exchange directly into leaf peroxisomes during greening and leaf peroxisomes are once again directly converted to glyoxysomes during senescence. The reversible transformations of microbodies are regulated at various levels, such as gene expression, splicing of the mRNA and degradation of microbody proteins. The regulatory mechanisms underlying this organelle differentiation are described.**

In higher plants, it is possible to regenerate a whole plant by cultivating a single vegetative cell. This ability, called "totipotency", is closely related to the flexibility of differentiation of higher plant cells. Functional transformation of organelles is frequently observed in higher plant cells, especially during the proliferation and differentiation of these cells. These transformations of organelles are induced by environmental changes such as light and temperature, and seem to be one of the characteristics of higher plant cells. Functional changes at the organelle level are the basis for differentiation of higher plant cells. The flexibility of differentiation of higher plant cells can be taken as a reflection of reversibility of the functional transformation of organelles.

We are conducting a study to elucidate the flexibility of differentiation and the totipotency of higher plant cells by clarifying the reversibility of organelle transformation clear at the molecular level. In this paper, we describe recent progress in understanding of the regulatory mechanisms underlying functional transformations of microbodies in higher plant cells.

Microbodies are ubiquitous organelles in eukaryotic cells, and are generally defined as organelles that contain catalase and at least one H_2O_2-producing oxidase (3, 21, 27, 35). In higher plants, microbodies can be categorized into at least three classes that are distinguishable in terms of function (15). [1] Glyoxysomes, which are found in germinating cotyledons of oil seeds, contain some enzymes required for the β-oxidation of fatty acids and the glyoxylate cycle, which participates in the mobilization of lipids. [2] Leaf peroxisomes are found in green leaves and function together with mitochondria and chloroplasts in photorespiratory glycolate metabolism. [3] Unspecialized microbodies with undefined metabolic roles are present in other organs, such as roots and stems. Although these three classes of microbodies are usually found in different organs, functional transformation of microbodies is observed in cotyledons of fatty seedlings under specific conditions.

[1] To whom correspondence should be addressed.
Phone: 81–564–55–7500, Fax: 81–564–55–7505

Functional Transformation of Microbodies during Greening and Senescence in Cotyledons of Fatty Seedlings

The functional transition of microbodies from glyoxysomes to leaf peroxisomes occurs in cotyledons of fatty seedlings, such as those of pumpkin and watermelon, during greening (3). An important question is whether the functional transition of microbodies proceeds in a linear and continuous fashion, or whether it proceeds in a discontinuous, two-step sequence. Two opposing hypotheses, i.e. the one-population and two population models, have been proposed (3). According to the former, glyoxysomes are directly transformed to leaf peroxisomes during greening of cotyledons, accompanying the insertion of newly synthesized leaf peroxisome-specific enzymes and the concomitant breakdown of glyoxysome-specific enzymes (4, 36). On the contrary, the two-population hypothesis postulates that glyoxysomes are broken down and leaf peroxisomes are newly synthesized *de novo* (16, 17). According to the one population hypothesis, as schematically illustrated in Figure 1, both glyoxysome-specific and leaf peroxisome-specific enzymes co-exist within a single microbody species throughout seedling growth ever after illumination. On the other hand, the two-population hypothesis suggests that the glyoxysome-specific and leaf peroxisome-specific enzymes are segregated and exist separately in ontogenically different microbodies, never coexisting in the same microbody during greening. Immunocytochemical analysis revealed that both glyoxysome-specific and leaf peroxisome-specific enzymes coexist in the microbody during the transitional stage, indicating that glyox-

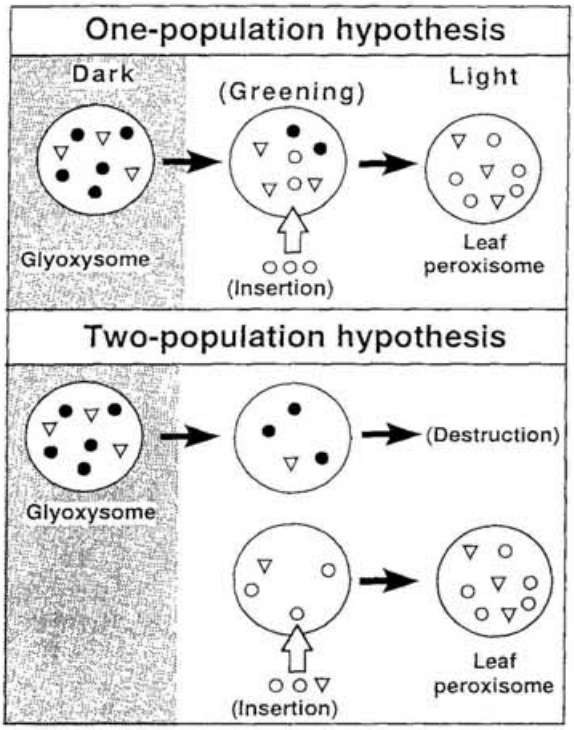


Fig. 1. Two hypothetical mechanisms proposed for the transition of glyoxysomes to leaf peroxisomes in fatty seedlings. (●), Glyoxysome-specific enzyme; (○), leaf peroxisome-specific enzyme; (▽), enzymes present in both glyoxysomes and leaf peroxisomes.

ysomes are directly transformed to leaf peroxisomes during greening (26, 34).

After greening, the cotyledons undergo senescence, which is accompanied by transfer of their stores of carbon and nitrogen to newly developing tissues. The reverse transition of microbodies from leaf peroxisomes

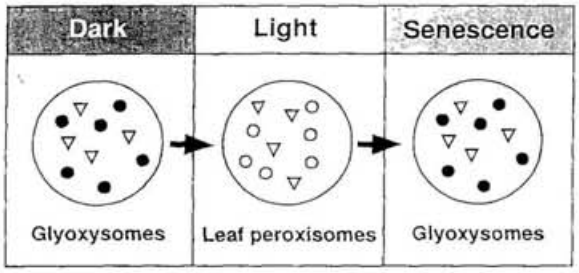


Fig. 2. Functional transition of microbodies during greening and senescence of fatty seedlings. (●), Glyoxysome-specific enzyme; (○), leaf peroxisome-specific enzyme; (▽), enzymes known to exist in both glyoxysomes and leaf peroxisomes.

to glyoxysomes occurs in the cotyledons during senescence (7). A similar immunocytochemical analysis has shown that leaf peroxisome-specific and glyoxysome-specific enzymes coexist in the microbody of senescing cotyledons (25). These findings revealed that leaf peroxisomes are directly transformed to glyoxysomes during senescence of cotyledons (Fig. 2).

Regulatory Mechanisms Underlying the Microbody Transition

During the microbody transition of fatty seedlings, no morphological differences in microbodies are observed. However, the compositions of the enzymes in microbodies are changed drastically during the microbody transition. The enzymes localized in microbodies can be divided into the following three categories. One is the glyoxysome-specific enzymes, such as malate synthase and citrate synthase which are members of the glyoxylate cycle. The second is leaf peroxisome-specific enzymes such as glycolate oxidase and hydroxypyruvate reductase, which function in glycolate metabolism of photorespiration. The third is the enzymes present in glyoxysomes and leaf peroxisomes such as catalase and the enzymes of β-oxidation cycle. Figure 3 shows schematically the developmental change in activities of these three microbody enzymes during greening. In dark-grown cotyledons, activities of glyoxysome-specific enzymes increase with the germination and decrease gradually after reaching a maximum level. In contrast, activities of leaf peroxisome-specific enzymes are at a low level in dark-grown cotyledons. When the seedlings are transferred to the light, activities of glyoxysome-specific enzymes decrease rapidly whereas those of leaf peroxisome-specific enzymes increase markedly. Developmental changes in the activities of enzymes present in both microbodies are similar to those of glyoxysome-specific enzymes. After exposure to light, the activities of glyoxysome-specific enzymes disappear, whereas those of enzymes present in both microbodies decrease but are still retained at a low level after completion of the microbody transition from glyoxysomes to leaf peroxisomes.

Therefore, the microbody transition observed during greening can be divided into at least the following three light-dependent phenomena. [1] The decrease and disappearance in the level of activities of glyoxysome-specific enzyme. [2] The appearance and accumulation in the level of activities of leaf peroxisome-specific enzymes. [3] The decrease in activities of enzymes present in both microbodies.

This paper is focused on the regulatory mechanisms underlying the microbody transition from glyoxysomes to leaf peroxisomes.

Regulation in the Level of Gene Expression

Northern blot analysis using cDNA for malate syn-

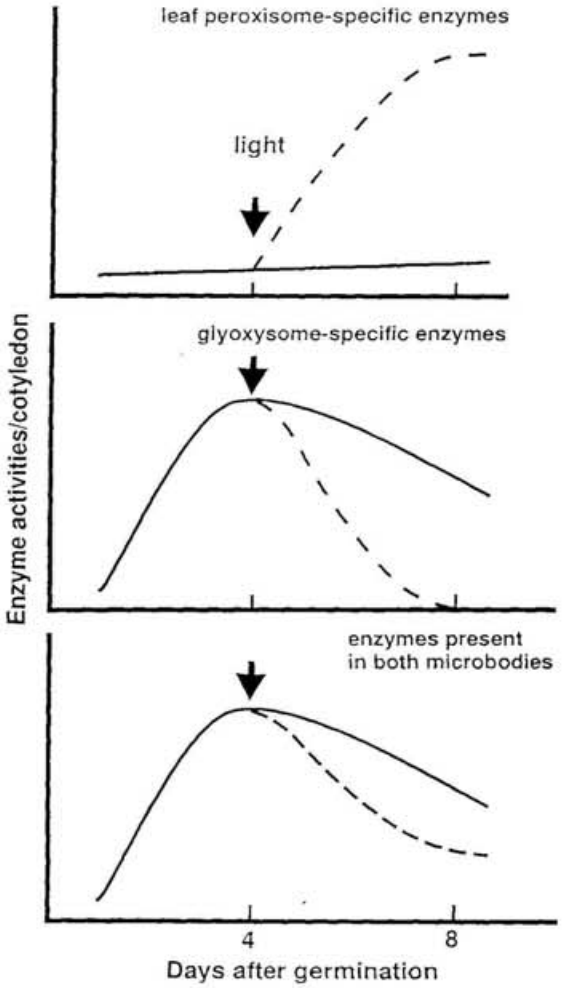


Fig. 3. Changes in enzyme activities for various microbody enzymes in cotyledonary tissues during germination and subsequent greening. Solid lines indicate enzyme activities found in cotyledons grown in darkness. Dotted lines indicate enzyme activities found in cotyledons grown under illumination after germination in darkness for 4 days. Arrows show the times at which cotyledons were exposed to light.

thase and citrate synthase revealed that mRNA for glyoxysome-specific enzymes increases during germination in darkness but decreases rapidly after the exposure to light and finally disappears (19, 24). It is clear that the microbody transition is caused partly by a decrease in the levels of the mRNA for glyoxysomal enzymes. In contrast, the amount of the mRNA for glycolate oxidase, a leaf peroxisomal enzyme, is low in dark-grown cotyledons, and increases markedly during greening (37).

During the senescence of cotyledons, the changes in the levels of mRNA for glyoxysome-specific enzymes and leaf peroxisome-specific enzymes are opposite to the changes that occur during greening. Gene expression of leaf-peroxisomal enzymes seems to be regulated by light and it has been reported that phytochrome is involved in the signal transduction (30). Gene expression of malate synthase, a glyoxysome-specific enzyme, is shown to be inhibited by sucrose (29). Therefore, there is a possibility that the microbody transition is regulated not only by light but also by the level of metabolites.

Regulation at the Level of Protein Transport to Microbodies

Microbody enzymes function after their transport to microbodies. Since the enzyme compositions and functions of glyoxysomes and leaf peroxisomes differ from each other, it was likely that the two types of microbodies possess different machineries for protein import.

Microbody proteins are synthesized in the cytosol on free polysomes and are transported post-translationally into microbodies. Two types of targeting signals to microbodies have been reported (Fig. 4). One type of targeting signal is part of the mature protein. One such signal, the tripeptide Ser-Lys-Leu, occurs at the C-terminal end has been identified as a targeting signal (for reviews, see (31, 32)). Ser-Lys-Leu and related amino acid sequences commonly function in mammals, insects, fungi, and plants (10). Glyoxysomal enzymes, such as malate synthase (5, 11, 24, 38) and isocitrate lyase (2, 6, 22, 39), and leaf peroxisomal enzymes, such as glycolate oxidase (37, 40) and hydroxypyruvate reductase (13), contain the targeting signal at their C-terminal ends.

To characterize the targeting signal, transgenic *Arabidopsis* plants that expressed a fussion protein composed of the C-terminal five amino acids of pumpkin malate synthase and a bacterial protein, β-glucuronidase (GUS) were generated. Immunocytochemical analysis of the transgenic plants revealed that the carboxy-terminal five amino acids of pumpkin malate synthase were sufficient for transport of the fusion protein into glyoxysomes in etiolated cotyledons, into leaf peroxisomes in green cotyledons and in mature leaves, and into unspecialized microbodies in roots, although the fusion protein was no longer transported into microbodies when SRL at the carboxyl terminus was deleted (12). Transport of proteins into glyoxysomes and leaf peroxisomes was also observed when the carboxy-terminal amino acids of the fusion protein were changed from SRL to SKL, SRM, ARL or PRL (12).

An ability of 24 different carboxy-terminal amino acid sequences to facilitate the transport of the fusion

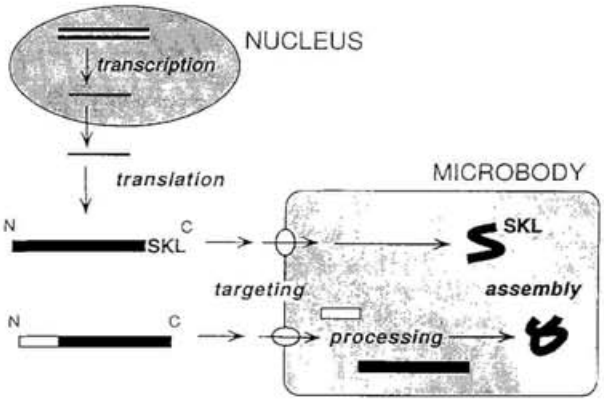


Fig. 4. Signals for protein transport to microbodies. Two types of signal are used for the transport of proteins into plant microbodies. N; amino terminus, C; carboxy terminus, SKL; SKL signal found in carboxy terminus of the microbody proteins.

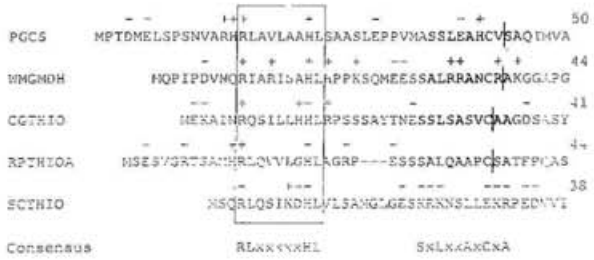


Fig. 5. Comparison of amino-terminal presequences found in microbody enzymes that are synthesized as high molecular weight precursors. PGCS; citrate synthase localized in pumpkin glyoxysomes. WGMDH; malate dehydrogenase localized in watermelon glyoxysomes. CTHIO; cucumber 3-keto-acyl CoA thiolase. RPTHIOA; thiolase A localized in rat peroxisomes. SCTHIO; thiolase localized in yeast peroxisomes. Box indicates consensus sequence of the signal for protein transport into microbodies, while shadow indicates amino acid sequences that are important for the processing of the presequences. Horizontal bars represent processing site of presequences. Cysteine residues at the processing sites are shown in bold. + and − indicate positively and negatively charged amino acids, respectively.

protein into leaf peroxisomes in green cotyledonary cells of transgenic *Arabidopsis* was examined (Hayashi, M., Aoki, M., Kondo, M. and Nishimura, M.; unpublished). Immunoelectron microscopic analysis revealed that carboxy-terminal tripeptide sequences of the form [C/A/S/P]-[K/R]-[I/L/M] function as a microbody-targeting signal, although tripeptides with proline at the first amino acid position and isoleucine at the carboxyl terminus show weak targeting efficiencies. All known microbody enzymes that are synthesized in a form similar in size to the mature molecule, except catalase, contain one of these tripeptide sequences at their carboxyl terminus.

A second type of targeting signal involved a cleavable N-terminal sequence. A small group of microbody proteins, such as 3-ketoacyl-coenzyme A (CoA) thiolase (14, 20, 28), malate dehydrogenase (9), glyoxysomal citrate synthase (gCS) (19) are synthesized as precursor proteins with larger molecular masses than those of the mature proteins. Each of these proteins has a cleavable presequence at its N-terminal end. Swinkels and colleagues (33) showed that the N-terminal presequence of 3-ketoacyl-CoA thiolase from rat liver functions as a targeting signal. The N-terminal region of gCS is highly homologous to those of other microbody proteins that are synthesized as larger precursors. There are two conserved sequences in their N-terminal regions (18) as shown in Figure 5. One such sequence is RL-X_5-HL, first recognized by de Hoop and Ab (8). The other sequence is SXLXXAXCXA, located at the cleavage site of the presequence. Transgenic *Arabidopsis* plants that expressed a fusion protein composed of the N-terminal region of gCS and GUS, were generated and their localization and processing were characterized by immunological and immunocytochemical analysis (18). The fusion protein was transported into functionally different microbodies, such as glyoxysomes, leaf peroxisomes and unspecialized microbodies and was subsequently processed. These observations indicated that the transport of gCS is mediated by its amino-terminal presequence and that the transport system is functional in all plant microbodies. Therefore, it is unlikely that glyoxysomes and leaf peroxisomes possess different targeting machineries.

A similar analysis of transgenic *Arabidopsis* plants that expressed fusion proteins with substituted amino acid residues in the two consensus sequences by site-directed mutagenesis shows that RL-X_5-HL functions in targeting to microbodies and SxLxxAxCxA plays a role in the processing of the N-terminal region (18).

Regulation at the Level of Splicing for mRNA

Hydroxypyruvate reductase belongs to the leaf-peroxisome-specific enzymes and is induced and accumulated in microbodies during greening. Two different cDNAs encoding hydroxypyruvate reductase were isolated from a cDNA library of pumpkin cotyledons (13). One of the cDNAs, designated HPR1, encodes a polypeptide of 386 amino acids, while the other, HPR2, encodes a polypeptide of 381 amino acids. Although the nucleotide and deduced amino acid sequences of these cDNAs are almost identical, the deduced HPR1 protein contains Ser-Lys-Leu at its carboxy-terminal end, which is known as a microbody-targeting signal, while the deduced HPR2 protein does not. Analysis of genomic DNA strongly suggests that HPR1 and HPR2 are produced by alternative splicing. These findings show that two different hydroxypyruvate reductase, HPR1

and HPR2, are localized in microbodies and accumulation of HPR1 in leaf peroxisomes during in cytosol, respectively, suggesting the possibility that the microbody transition may be regulated by alternative splicing.

Regulation at the Level of Protein Degradation

The decrease and disappearance of mRNA for glyoxysome after the exposure of light prevent to *de novo* synthesis of glyoxysome-specific enzymes. However, preexisiting glyoxysome-specific enzymes need to be degraded during greening. The degradation of glyoxysome-specific enzymes has been observed in protein import experiments *in vitro*. Malate synthase, a glyoxysome-specific enzyme, synthesized in *in vitro* transcription-translation are post-translationally imported into leaf peroxisomes and glyoxysomes as well as into microbodies in the transition stage (23). Imported malate synthase molecules are degraded only in the microbodies in the transition stage. This finding suggests that during the microbody transition from glyoxysomes to leaf peroxisomes, a degradation system for glyoxysome-specific enzyme is induced in the microbodies. However, the identity of protease(s) which catalyze the degradation is not known. It has been reported that the protease that catalyzes the degradation of insulin is found in mammals and is localized in microbodies although the substrates for the protease in microbodies are not clear (1). Further characterization of the degradation system is crucial for clarifying the regulatory mechanism underlying the microbody transition.

The regulatory mechanisms of the microbody transition between glyoxysomes and leaf peroxisomes are summarized in Figure 6. This organelle differentiation is regulated at various levels such as gene expression, splicing of mRNA and degradation of microbody proteins.

Recently, two proteins in glyoxysomal membranes with molecular masses of 31 kDa and 28 kDa were purified and characterized (42). The former was shown to be a novel ascorbate oxidase (41). It was found that the amounts of these membrane proteins decreased during the microbody transition from glyoxysomes to leaf peroxisomes and that the large one was retained in leaf peroxisomes, whereas the small one could not be found in leaf peroxisomes after completion of the microbody transition. The results clearly showed that membrane proteins in glyoxysomes change dramatically during the microbody transition, as do the enzymes in the matrix.

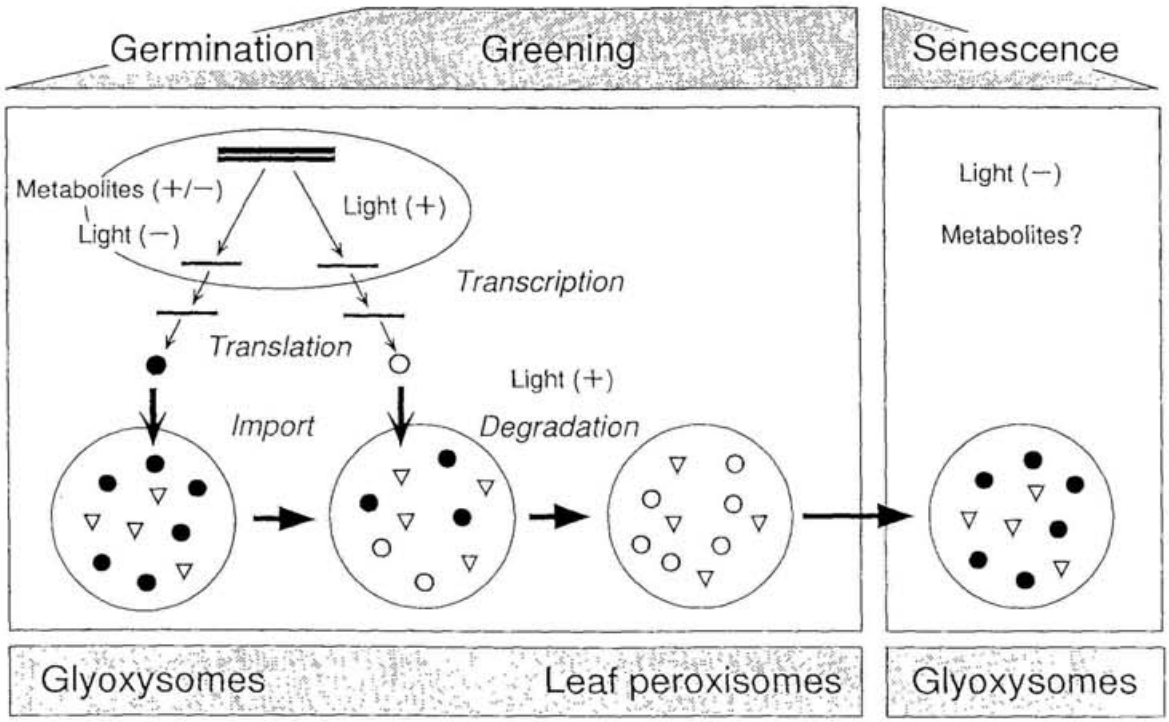


Fig. 6. Regulatory mechanisms underlying the microbody transition during greening and senescence of fatty seedlings. (●), Glyoxysome-specific enzymes; (○), leaf peroxisome-specific enzymes; (▽), enzymes present in both glyoxysomes and leaf peroxisomes.

The mechanisms for degradation of these membrane proteins still remain to be solved.

Most studies on microbody transitions have focussed on the transition from glyoxysomes to leaf peroxisomes during greening. However, the transformation of microbodies from leaf peroxisomes to glyoxysomes is observed during senescence of the cotyledons. How are these completely opposite developmental changes in microbody enzymes regulated during greening and senescence? Further analysis of regulation of the microbody transition during senescence is needed to characterize the mechanism of reversibility of the microbody transition in cotyledons.

Acknowledgements. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sport and Culture of Japan (nos. 07456053, 06248230 and 06261239) the NIBB program for Molecular Mechanisms of Stress Responses and from the Nissan Science Foundation (Tokyo, Japan).

REFERENCES

1. Authier, F., Bergeron, J.J.M., Ou, W.J., Rachubinski, R.A., Posner, B.I., and Walton, P.A. 1995. Degradation of the cleaved leader peptide of thiolase by a peroxisomal proteinase. *Proc. Natl. Acad. Sci. USA*, **92**: 3859–3863.
2. Beeching, J.R. and Northcote, D.H. 1987. Nucleic acid (cDNA) and amino acid sequences of isocitrate lyase from castor bean. *Plant. Mol. Biol.*, **8**: 471–475.
3. Beevers, H. 1979. Microbodies in higher plants. *Annu. Rev. Plant Physiol.*, **30**: 159–193.
4. Betshe, T. and Gerhardt, B. 1978. Apparent catalase synthesis in sunflower cotyledons during the change in microbody function. A mathematical approach for the quantitative evaluation of density-labeling data. *Plant Physiol.*, **62**: 590–597.
5. Comai, L., Baden, C.S., and Harada, J.J. 1989. Deduced sequence of a malate synthase polypeptide encoded by a subclass of the gene family. *J. Biol. Chem.*, **264**: 2778–2782.
6. Comai, L., Dietrich, R.A., Maslyar, D.J., Baden, C.S., and Harada, J.J. 1989. Coordinate expression of transcriptionally regulated isocitrate lyase and malate synthase genes in *Brassica napus* L. *Plant Cell*, **1**: 293–300.
7. De Bellis, L., Tsugeki, R., and Nishimura, M. 1991. Glyoxylate cycle enzymes in peroxisomes isolated from petals of pumpkin (*Cucurbita* sp.) during senescence. *Plant Cell Physiol.*, **32**: 1227–1235.
8. De Hoop, M.J. and Ab, G. 1992. Import of proteins into peroxisomes and other microbodies. *Biochem. J.*, **286**: 657–669.
9. Gietl, C. 1990. Glyoxysomal malate dehydrogenase from watermelon is synthesized with an amino-terminal transit peptide. *Proc. Natl. Acad. Sci. USA*, **87**: 5773–5777.
10. Gould, S.J., Keller, G.-A.A., Schneider, M., Howell, S.H., Garrad, L.J., Goodman, J.M., Distel, B., Tabak, H., and Subramani, S. 1990. Peroxisomal protein import is conserved between yeast, plants, insects and mammals. *EMBO J.*, **9**: 58–90.
11. Graham, I.A., Smith, L.M., Brown, J.W., Leaver, C.J., and Smith, S.M. 1989. The malate synthase gene of cucumber. *Plant Mol. Biol.*, **13**: 673–684.
12. Hayashi, M., Aoki, M., Kato, A., Kondo, M., and Nishimura, M. 1996. Transport of chimeric proteins that contain a carboxy-terminal targeting signal into plant microbodies. *Plant J.*, **10**: 225–234.
13. Hayashi, M., Tsugeki, R., Kondo, M., Mori, H., and Nishimura, M. 1996. Pumpkin hydroxypyruvate reductases with and without a putative C-terminal signal for targeting to microbodies may be produced by alternative splicing. *Plant. Mol. Biol.*, **30**: 183–189.
14. Hijikata, M., Wen, J.K., Osumi, T., and Hashimoto, T. 1990. Rat peroxisomal 3-ketoacyl-CoA thiolase gene. Occurrence of two closely related but differentially regulated genes. *J. Biol. Chem.*, **265**: 4600–4606.
15. Huang, A.H.C., Trelease, R.N., and Moore, T.S. 1983. *Plant peroxisomes*. Academic Press, New York, NY.
16. Kagawa, T. and Beevers, H. 1975. The development of microbodies (glyoxysomes and leaf peroxisomes) in cotyledons of germinating seedlings. *Plant Physiol.*, **68**: 845–850.
17. Kagawa, T., Load, J.M., and Beevers, H. 1975. Lecithin synthesis during microbody biogenesis of watermelon seedlings. *Arch. Biochem. Biophys.*, **167**: 45–53.
18. Kato, A., Hayashi, M., Kondo, M., and Nishimura, M. 1996. Targeting and processing of a chimeric protein with the amino-terminal presequence of the precursor to glyoxysomal citrate synthase. *Plant Cell*, **8**: 1601–1611.
19. Kato, A., Hayashi, M., Mori, H., and Nishimura, M. 1995. Molecular characterization of a glyoxysomal citrate synthase that is synthesized as a precursor of higher molecular mass in pumpkin. *Plant. Mol. Biol.*, **27**: 377–390.
20. Kato, A., Hayashi, M., Takeuchi, Y., and Nishimura, M. 1996. cDNA cloning and expression of a gene for 3-ketoacyl-CoA thiolase in pumpkin cotyledons. *Plant. Mol. Biol.*, **31**: 843–852.
21. Lazarow, P. and Fujiki, Y. 1985. Biogenesis of peroxisomes. *Annu. Rev. Cell Biol.*, **1**: 489–530.
22. Mano, S., Hayashi, M., Kondo, M., and Nishimura, M. 1996. cDNA cloning and expression of the isocitrate lyase gene in pumpkin cotyledons. *Plant Cell Physiol.*, **37**: 941–948.
23. Mori, H. and Nishimura, M. 1989. Glyoxysomal malate synthase is specifically degraded in microbodies during greening of pumpkin cotyledons. *FEBS Lett.*, **244**: 163–166.
24. Mori, H., Takeda, Y.Y., Hara, N.I., and Nishimura, M. 1991. Pumpkin malate synthase: Cloning and sequencing of the cDNA and Northern blot analysis. *Eur. J. Biochem.*, **197**: 331–336.
25. Nishimura, M., Takeuchi, Y., De Bellis, L., and Hara-Nishimura, I. 1993. Leaf peroxisomes are directly transformed to glyoxysomes during senescence of pumpkin cotyledons. *Protoplasma*, **175**: 131–137.
26. Nishimura, M., Yamaguchi, J., Mori, H., Akazawa, T., and Yokota, S. 1986. Immunocytochemical analysis shows that glyoxysomes are directly transformed to leaf peroxisomes during greening of pumpkin cotyledons. *Plant Physiol.*, **80**: 313–316.
27. Olsen, L.J. and Harada, J.J. 1995. Peroxisomes and their assembly in higher plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **46**: 123–146.
28. Preisig-Muller, R. and Kindl, H. 1993. Thiolase mRNA translated *in vitro* yields a peptide with a putative N-terminal presequence. *Plant Mol. Biol.*, **22**: 59–66.
29. Sarah, C.J., Graham, I.A., Reynolds, S.J., Leaver, C.J., and Smith, S.M. 1996. Distinct cis-acting elements direct the germination and sugar responses of the cucumber malate synthase gene. *Mol. Gen. Genet.*, **250**: 153–161.

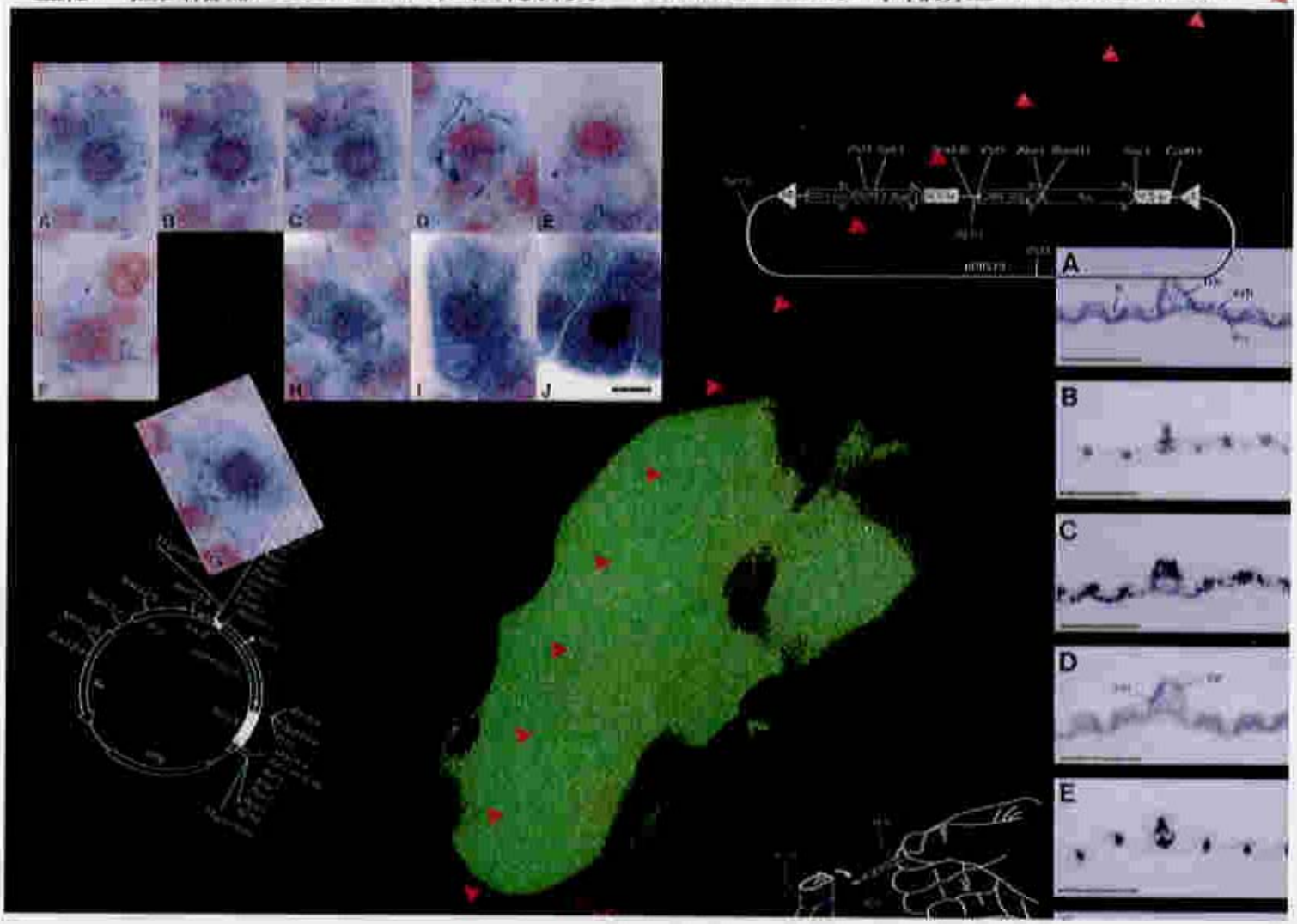
30. Schwartz, B.W., Sloan, J.S., and Becker, W.M. 1991. Characterization of genes encoding hydroxypyruvate reductase in cucumber. *Plant Mol. Biol.*, **17**: 941–947.
31. Subramani, S. 1992. Targeting of proteins into the peroxisomal matrix. *J. Membr. Biol.*, **125**: 99–106.
32. Subramani, S. 1993. Protein import into peroxisomes and biogenesis of the organelle. *Annu. Rev. Cell Biol.*, **9**: 445–478.
33. Swinkels, B.W., Gould, S.J., Bodnar, A.G., Rachubinski, R.A., and Subramani, S. 1991. A novel, cleavable peroxisomal targeting signal at the amino-terminus of the rat 3-ketoacyl-CoA thiolase. *EMBO J.*, **10**: 3255–3262.
34. Titus, D.E. and Becker, W.M. 1985. Investigation of the glyoxysome-peroxisome transition in germinating cucumber cotyledons using double-label immunoelectron microscopy. *J. Cell. Biol.*, **101**: 1288–1299.
35. Tolbert, N.E. 1981. Metabolic pathways in peroxisomes and glyoxysomes. *Annu. Rev. Biochem.*, **50**: 133–157.
36. Trelease, R.N., Becker, W.N., Gruber, P.I., and Newcomb, E.M. 1971. Microbodies (glyoxysomes and peroxisomes) in cucumber cotyledons. Correlative biochemical and ultrastructural study in light- and dark-grown seedlings. *Plant Physiol.*, **48**: 461–475.
37. Tsugeki, R., Hara-Nishimura, I., Mori, H., and Nishimura, M. 1993. Cloning and sequencing of cDNA for glycolate oxidase from pumpkin cotyledons and Northern blot analysis. *Plant Cell Physiol.*, **34**: 51–57.
38. Turley, R.B., Choe, S.M., Ni, W., and Trelease, R.N. 1990. Nucleotide sequence of cottonseed malate synthase. *Nucleic Acids Red.*, **18**: 3643.
39. Turley, R.B., Choe, S.M., and Trelease, R.N. 1990. Characterization of a cDNA clone encoding the complete amino acid sequence of cotton isocitrate lyase. *Biochim. Biophys. Acta*, **1049**: 223–226.
40. Volokita, M. and Somerville, C.R. 1987. The primary structure of spinach glycolate oxidase deduced from the DNA sequence of a cDNA clone. *J. Biol. Chem.*, **262**: 15825–15828.
41. Yamaguchi, K., Mori, H., and Nishimura, M. 1995. A novel isoenzyme of ascorbate peroxidase localized on glyoxysomal and leaf peroxisomal membranes in pumpkin. *Plant Cell Physiol.*, **36**: 1157–1162.
42. Yamaguchi, K., Takeuchi, Y., Mori, H., and Nishimura, M. 1995. Development of microbody membrane proteins during the transformation of glyoxysomes to leaf peroxisomes in pumpkin cotyledons. *Plant Cell Physiol.*, **36**: 455–464.

細胞工学別冊 植物細胞工学シリーズ 6

植物の細胞を観る 実験プロトコール

遺伝子発現から細胞内構造・機能まで

監修 福田裕穂 東京大学理学系研究科生物科学専攻／西村幹夫 名古屋大学大学院生命農学研究科／中村研三 名古屋大学大学院生命農学研究科



3-16

GFPを用いたタンパク質輸送の観察法②培養細胞を材料とする方法

植物体の内部構造を観察するためには，通常，組織切片を作製し，適当な染色をした後，顕微鏡で観察しなければならない．しかし，培養細胞の場合は，細胞内オルガネラに局在するタンパク質を，切片を作製することなく容易に観察できる．タバコ培養細胞BY-2株(以降，BY-2と略す)は，生育速度が速く均一な細胞集団で，しかも形質転換も容易なことから，特定遺伝子の機能解析に用いられている．ここでは，このBY-2を材料とし，レポータータンパク質として，近年よく用いられているGreen Fluorescent Protein(GFP)とのキメラタンパク質を用いた緑葉ペルオキシソームタンパク質輸送の観察の手法をまとめた．GFPの諸性質については本書3-5を参照していただきたい．

原　理

タンパク質輸送を解析する手法の1つとして，レポータータンパク質遺伝子とのキメラ遺伝子を作製し，その遺伝子産物の細胞内局在性を観察する方法がとられている．特に，β-glucuronidase(GUS)やluciferaseは基質を加えることにより，その遺伝子産物の細胞内局在性を組織化学染色により観察できるという利点から，レポーター遺伝子として頻繁に用いられてきた．一方，GFPは，基質を加える必要がなく青色光をあてるだけで，しかも生きた状態で解析が可能であることから，近年，生体蛍光マーカーとして注目を浴びている．ここでは，緑葉ペルオキシソームタンパク質であるヒドロキシピルビン酸レダクターゼ1(HPR1)*1の輸送シグナル(PTS1)*2およびサイトソルに局在するヒドロキシピルビン酸レダクターゼ2(HPR2)*1のC末端をGFPにつなげたキメラタンパク質をBY-2で発現させ，その細胞内局在性を観察する方法を示す(図1)．

準　備

器具・装置

- 遠心機：15ml/コニカルチューブを回せるもの（TOMY SEIKO；MODEL LC60など）
- インキュベーター
- 蛍光顕微鏡（オリンパス；落射蛍光装置BX-FLAなど）
- 共焦点レーザー顕微鏡（オリンパス；LSM-GB20など）
- EGカットテープ（共和）

試薬

- BY-2培地（培地組成とBY-2*3の培養方法は付録1を参照）

*1　ヒドロキシピルビン酸レダクターゼは，光呼吸におけるグリコール酸経路の一部をつかさどる酵素で，カボチャの場合は1つの遺伝子からalternative splicingにより合成されるHPR1，HPR2が存在する．それらのタンパク質の一次構造は，C末端のアミノ酸のみ異なり，HPR1は，そのC末端に存在する輸送シグナルのために緑葉ペルオキシソームに輸送されるが，HPR2は輸送シグナルがないためサイトソルに留まる．

*2　PTS (peroxisome targeting signal；ペルオキシソームタンパク質の輸送シグナル)は，主に2種類が知られている．そのうち，サイトソルで翻訳される前成熟型タンパク質と同じ分子量で合成されるものは，比較的よく保存されたC末端のトリペプチド(Ser-Ala-Lys, Arg-His-Leu)によってペルオキシソームへ輸送される．

*3　BY-2はタバコ(*Nicotiana tabacum* cv. Bright Yellow No.2)の葉生え由来の培養細胞で，その懸濁培養細胞は2～8個の細胞から成り，ほとんどすべての細胞が分裂，増殖するため全体の増殖時間が早い．アメリカではNTあるいはNT-1と呼ばれている．

・*Agrobacterium*培地（YEB培地）

0.5%	beef extract
0.1%	yeast extract
0.5%	polypeptone
0.5%	sucrose
0.05%	$MgSO_4$

・硫酸カナマイシン（明治製菓；硫酸カナマイシン明治，筋注用1g入）

・カルベニシリンナトリウム（ナカライテスク；カルベニシリン二ナトリウム）

方　法

▶ 1．アグロバクテリウムの構造

図2に示すバイナリープラスミド（pMAT-SGFP-H1，pMAT-SGFP-H2*4）をもつ*Agrobacterium* EHA101株を用いた例を示す．このプラスミドは，カナマイシン耐性遺伝子の下流に，カリフラワーモザイクウイルス35Sプロモーター，その下流に*Bgl* IIと*Eco*R I部位を付加した改変sGFP（sGFP-BE）をつなぎ，この*Bgl* IIと*Eco*R I部位の間に，HPR1とHPR2のC末端アミノ酸10個を付加した構造*5になっている．

*4　コンストラクトのもととなるpMAT037は，名古屋大学大学院 生命農学研究科の中村研 二先生より，SGFP-TYGは，静岡県立大学大学院生活健康科学研究科の丹羽康夫先生より分与していただいた．

*5　GFPとの融合タンパク質を作製する場合，目的のタンパク質をGFPにすぐつなげるのではなく，GFPの立体構造を妨げないようにするために，リンカーとして2～5個くらいのアミノ酸を挟んだ方がよい．筆者らは，アスパラギン酸とロイシンを挟んでいる（図2参照）．

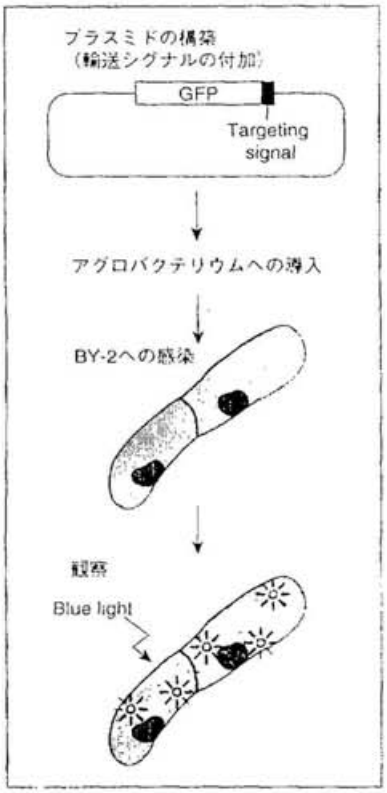


図1　キメラGFP遺伝子のBY-2への導入の概略

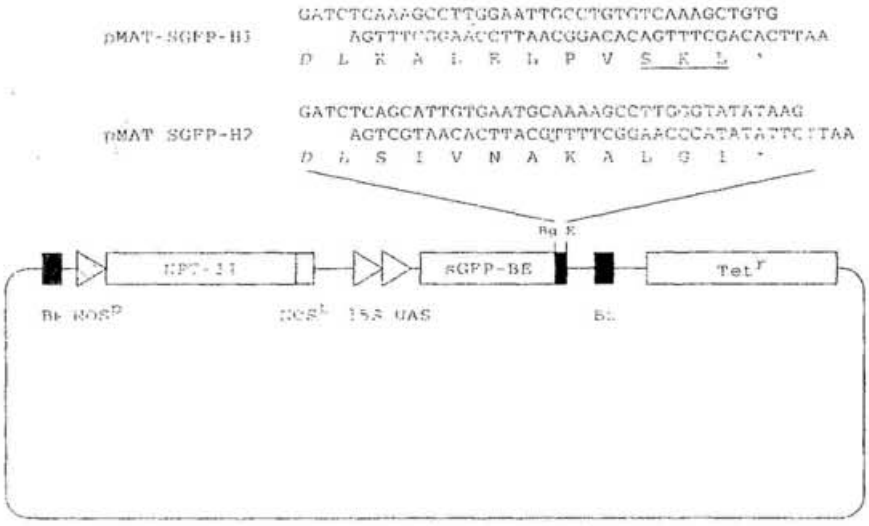


図2　導入したプラスミドの構造

GFPの後ろに組み込んだ塩基配列および対応するアミノ酸を塩基配列の下に示した．下線部はPTS（peroxisome targeting signal）を，*はストップコドンを示す．斜体のアミノ酸はGFPでもHPRでもない配列でリンカー部位である．NOS^P：ノパリン合成酵素プロモーター，NPT-II：カナマイシン耐性遺伝子，NOS^T：ノパリン合成酵素ターミネーター，35S UAS：カリフラワーモザイクウイルス35Sプロモーター，Tet^r：テトラサイクリン耐性遺伝子，Bg：*Bgl* II，E：*Eco*R I．

▶ 2. アグロバクテリウムの感染

1) 9cmプラスチックシャーレに，植え継いで3日目のBY-2培養液4m*l* を移す．

↓

2) 30℃で一夜培養[*6]した*Agrobacterium* EHA101(pMAT-SGFP-H1，pMAT-SGFP-H2)100 μl を加える．

↓

3) シャーレを軽く回して混合し，シャーレ全体に培養液を広げた後，EGカットテープでシールし，26℃，暗所で42～48時間，静置培養する．[*7]

▶ 3. BY-2細胞の洗浄とスクリーニング

1) シャーレのBY-2細胞を15m*l* コニカルチューブに移す．

↓

2) 5m*l* のBY-2培地を加え，ピペットで吸い上げては押し出す操作(ピペッティング)を10回繰り返す．[*8]

↓

3) 800rpmで1分間遠心し，BY-2細胞を沈殿させる．[*9]

↓

4) 上清を吸い取り，BY-2培地を5m*l* 加え，2)～3)と同じ操作を繰り返す．

↓

5) 4)を5回繰り返した後，BY-2培地を沈殿の10倍量(0.2m*l* pcv/m*l* [*10])加える．

↓

6) このうち1m*l* を，形質転換用固形培地(BY-2固形培地にカナマイシンとカルベニシリンを加えたもの)[*11]に均一に広げる．

↓

7) 余分な液体を除いた後，EGカットテープでシールする．

↓

8) 28℃で10日～3週間培養する．

↓

9) ピンセットで摘めるくらいの形質転換コロニーが出てきたら，同じ組成の形質転換用固形培地に移し，再び10日～3週間培養する．[*12]

↓

10) コロニーが大きくなったらその一部を掻き取ってマイクロチューブ中の水に懸濁し，その一部を蛍光顕微鏡でB(Blue)励起の下で観察する．[*13]

↓

11) 強い光が観察されるコロニーを選抜し，同組成の形質転換用固形培地に塗布する．

↓

12) コロニーが生えてきたら95m*l* の液体培地(抗生物質は固形培地の半分の濃度)に移し，数日間振盪培養する．

↓

13) ほぼ飽和に達した時点[*14]で，新しい液体培地に5m*l* 継代する．

↓

14) 徐々に除菌用抗生物質の濃度を下げ，継代量を減らしていき生育速度が一定になるまで調製する．

＊6 L-brothでも生育可能だが，培地に含まれるNaClがBY-2にダメージを与えるので，YEB培地またはAB sucrose培地で培養するのが望ましい．

＊7 この時，きちんと水平に保つことが大切である．培地が片寄ると，培地に触れていないBY-2は乾燥して死に，多量の培地に沈んでいるものは窒息死する．

＊8 このステップは細胞を均一にするとともに，*Agrobacterium* を取り除くために行う．

＊9 この時，沈殿が1～1.5m*l* の範囲にあることが望ましい．

＊10 pcv=packed cell volume

＊11 形質転換体選抜用抗生物質として硫酸カナマイシンを200mg/*l* で，*Agrobacterium* 除去のための抗生物質としてカルベニシリンナトリウムを500mg/*l* で用いる．

＊12 形質転換がうまくいっていれば，1枚のプレートあたり数千個のコロニーが得られるはずである．このまま，掻き取って懸濁培養にもっていく方法もあるが，ヘテロな細胞の集団になってしまうので，できれば強く発現している細胞を選んだ方が，後々の解析が楽である．

＊13 実際には，10)の段階で余ったコロニーを覗いても観察可能である．sGFPの励起波長は490nmの単一のピークである．筆者らは，オリンパス社製のU-MNIB(励起波長470～490nm，吸収波長515nm以上)またはPhotometrics社製のマルチフィルタ(80P000 PINKEL602：励起波長490nm，吸収波長510nm以上)を使用している．

＊14 wild typeで1週間たった液体培地と同じくらいに増殖していればよい．条件に左右されるので，研究室によって多少目安が異なる．

▶ 4. GFPの観察

上記の方法に従えば、安定な形質転換体が得られるが、今回用いているsGFPは蛍光強度が大きいため、安定な形質転換体が得られる前にもGFPの光は観察可能である。筆者らは、コロニーの段階で観察できることを確認しているが、この場合、早ければ*Agrobacterium*をかけてから10日でGFPの光が検出可能である。

1) コロニーの場合はその一部を掻き取り、H_2Oまたは終濃度0.5Mになるようにマンニトールを加えたBY-2培地に懸濁する。液体培養の場合は、その一部をそのまま用いる。*15

↓

2) 試料をスライドグラス上にとり、カバーグラスを載せ観察する（結果例：図3）。*16, 17→ TS3

コメント

1. BY-2細胞を1 μg/mlのDAPIで染色すると、核がUV照射下で青色光として観察できる。また、各オルガネラのマーカー酵素をRhodamineなどで染色することによってGFPとの二重染色も可能である。
2. 蛍光顕微鏡下では細胞の立体構造のため、前後のぼやけた光をバックグラウンドとして拾ってしまうため、きれいな像が得られない場合がある。その際には、共焦点レーザー顕微鏡を用いることでバックグラウンドを消すことができる。
3. 筆者らは、解析システムとしてPhotometrics社製のPXL1400（セミナ…ト

*15 サンプリングしてすぐに観察する場合であれば、水に懸濁して観察すればよいが、しばらくの間、細胞を維持したい場合は、浸透圧の問題からBY-2培地に終濃度0.5Mになるようにマンニトールを加えた溶液に懸濁した方がよい。懸濁培養の場合も観察してみてゴミが多いようであれば、遠心して沈殿にした後、再懸濁した方がきれいな像が得られる場合がある。

*16 GFPの観察像は使用するフィルターにより大きく影響を受ける。使用するフィルターによっては葉緑体自身が蛍光を持つ可能性もある。筆者らも、オリンパス社製のU-MNIBとPhotometrics社製のマルチ・フィルター（91000 PINKEL）を使用しているが、後者の方が葉緑体自身のバックグラウンドが少なくGFPの観察には適していると思われる。

*17 例えば、筆者らが観察しているミトコンドリアなど、目的とするオルガネラによっては高倍率で確認した方がよい場合もある。

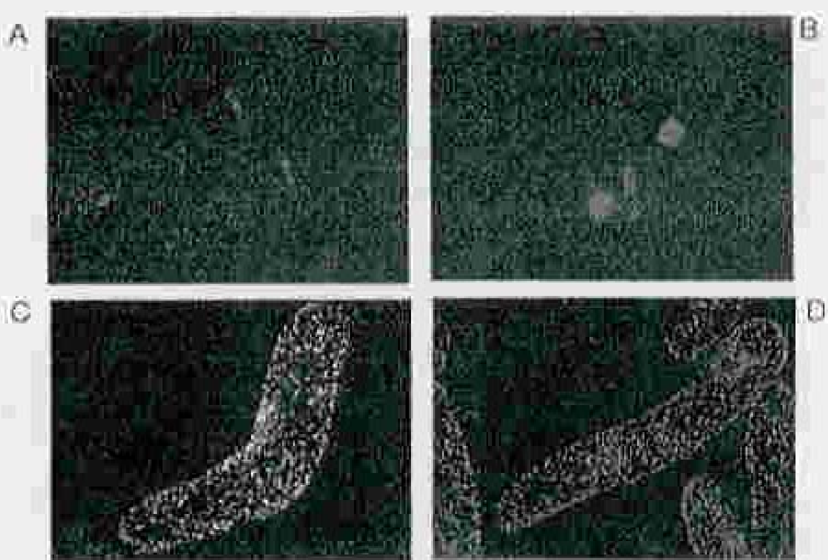


図3 BY-2におけるGFPの蛍光観察像（口絵参照）

A、C、D：pMAT-SGFP-HL、B：pMAT-SGFP-IIを導入したBY-2。A、Bは明視野下で観察したもの。C、DはpMAT-SGFP-HLを導入したBY-2の形質転換体のステレオ像。pMAT-SGFP-HLを導入した場合は、C末端のHDELのためペルオキシソームへ輸送され、ペルオキシソームで光が観察される。pMAT-SGFP-IIの場合は、サイトゾルに留まるため細胞全体が光って見える。GFPは核へ移行する傾向があるため、核に光が集まって見える場合もある。

ロン社取扱)を使用している．これは，冷却CCDカメラを通して取り込んだ画像を，バックグラウンドの消去，三次元像の構築，二重染色の重ね合わせなど種々の解析が可能なシステムで共焦点像も作製可能である．

④筆者らは，Dr. Haseloffから分与していただいたmGFP4も同じ方法でBY-2に導入して蛍光観察した．mGFP4は当初，野生型GFPをシロイヌナズナに導入するとミススプライシングが起こり，クリプティックなイントロンが切り出され活性がなくなることが報告されたため[5]，Haseloffらがクリプティックイントロン部位を改変し，植物においても活性をもつようにしたものである．この場合も，緑葉ペルオキシソームでの光が観察されたが，sGFPに比べるとはるかに蛍光強度が小さいため，今回筆者らが用いたような改変した蛍光強度の大きいGFPを使用することをお薦めする．

⑤タンパク質の細部内局在性を観察する場合，間接蛍光抗体法という手法もある．この場合，細胞内オルガネラに集積しているタンパク質は比較的容易に検出できるが，サイトソルタンパク質の中には，30 μm以上のかなり厚い切片でないと検出できないものがある(おそらく，濃度が薄いことが原因であると考えられる)．**3-1**で紹介されている0.5～1 μmの薄い切片を用いた方法や，免疫電顕観察では，緑葉ペルオキシソームタンパク質であるヒドロキシピルビン酸レダクターゼ1 (HPR1) は検出できても，サイトソルに局在するヒドロキシピルビン酸レダクターゼ2 (HPR2) は検出できない．また，間接蛍光抗体法は，かなりステップを踏んだ手法であるので，今回，紹介したGFPを用いた非破壊的な観察法は，簡便で有力な手法といえよう．

トラブルシューティング

TS 1　共焦点像を数枚撮影する場合，時によってカバーグラスとスライドグラスの間に空気が入ってきて，対象物が移動してしまうことがある．

筆者らは，それを避けるためカバーグラスの周りをマニキュアで固めている．マニキュアは市販されているもので構わないが，塗りすぎると逆にカバーグラスの下に入ってきて，蛍光を発する場合があるので注意する．

◆参考文献

1) An G. : Binary Ti vectors for plant transformation and promoter analysis. Method in Enzymology vol. 153, 292-305, Academic Press (1987)
2) 松岡　健 : 第8回 基礎生物学研究所 バイオサイエンストレーニングコース, 69-71 (1993)
3) Chiu W-L., Niwa Y. et al. : Engineered GFP as a vital reporter in plants. Current Biology 6, 325-330 (1996)
4) Hayashi, M., Tsugeki, R. et al. : Pumpkin hydroxypyruvate reductases with and without a putative C-terminal signal for targeting to microbodies may be produced by alternative splicing. Plant Mol. Biol. 30, 183-189 (1996)
5) Haseloff, J. & Amos, B. : GFP in plants. Trends Genet. 11, 328-329 (1995)

(真野昌二　基礎生物学研究所 細胞生物学研究系細胞機構研究部門・総合研究大学院大学 生命科学研究科)