Artificially-Synthesized Intergeneric Chimera between *Brassica oleracea* and *Raphanus sativus* by *In Vitro* Grafting

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Abstract

The first intergeneric chimeras between radish and cabbage were successfully synthesized by *in vitro* grafting method. Regenerated seventy seven chimeras from the original sectorial chimera were investigated on morphological, physiological natures comparing with other interspecific chimeras of *Brassica*. Chimeral structures were justified and totally classified based on the morphological characteristics, isozymatic band patterns and PCR analysis. In those chimeras, putative four-layered chimeral plants were also found for two vegetatively-propagated generations with general three-layered chimeras. Physiological interactions were typically recognized such as in flowering date, pollen fertility, and pod and seed set. However, genetic changes were not found in the progeny derived from the crosses with both parents.

1. Introduction

We have been studying graft transformation in higher plants (Hirata *et al.* 1995; Taller *et al.*, 1998) and extending the induction of genetic changes by using the cell to cell interaction in chimeral plants.

For the purpose *in vivo* grafting (Hirata *et al.*, 1992) and *in vitro* graft culture methods (Noguchi *et al.*, 1992) were developed and four kinds of interspecific chimeras were artificially synthesized in *Brassica* crops. Morphogenic, physiologic and genetic interactions between different cells and tissues have been studied using these chimeras. Genetic as well as morphogenic and physiological interactions were observed. Genetic changes could be obtained in the progeny derived from the crosses between chimeras and their parents. Variant lines that posse cytoplasmic male sterility were found among the progeny obtained by crossing the chimera between cv. Komatsuna (*B. campestris*) and cv. Ruby ball (*B. oleracea*) with Komatsuna. In the CMS variants the mitocondrial DNA of Komatsuna and Ruby ball (cabbage) is appeared to be recombined whereas the nuclear genomes were not largely changed (Takeda *et al.*, 1992; Hirata *et al.*, 1997).

Morphological and physiological interactions were variously observed along with the genetic variations (Noguchi and Hirata, 1994; Hirata *et al.*, 1994).

In the present study we attempted to make further intergeneric *in vitro* graft chimeras between *B. oleracea* and *Raphanus sativus*, and to investigate the morphogenetical nature of the resulting chimeras, the first success using an *in vitro* grafting method.

In the present paper, some interactions between different tissues in the intergeneric chimeras are described.

2. Materials and Methods

Red cabbage cultivar "Ruby ball" in *Brassica oleracea* and radish cultivar "Miryokuna" (*Raphanus sativus*) which is cultivated as a leaf vegetable in Japan served as synthesizing materials.

To synthesize chimera we applied the *in vitro* approach-grafting method to young seedling just after the emergence (Noguchi *et al.*, 1992). Twenty grafts were done and the resulting chimeral apical part was subcultured on the modified B5 medium (Gamborg *et al.*, 1968) supplemented with $3.75\,\mathrm{g}\,l^{-1}$ $CaCl_2 \cdot 2H_2O$ and 1ppm 6-benzylaminopurine (BA). Regenerated chimeral apical shoots were transplanted onto the half strength of MS medium (Murashige and Skoog, 1962) without hormones for rooting. Seventy seven regenerates were transferred

into a soil pot and acclimatized in a greenhouse. Morphogenetical and physiological analyses were continued during growing stages. To identify cell layer and tissue constitutions we made transversal leaf sections using a microslicer (Osaka EM, DTK 1000) and observed the leaf tissue structure and anthocyanin pigmentation pattern. In addition, PCR analysis was done for mitochondrial DNA by using the applicable primer of *atp6*. Primers 5'-ATCTTT-CGGCACCTTGATCG-3' at the sense side and 5'-AACACTACTACTCTCAATCCCTCG-3' at the anti-sense side were used for PCR. In PCR analysis, amplified condition of 43 cycles of 94 ℃ for denaturation, 72 ℃ for extension, 42 ℃ for annealing was adopted by using X Model PC-700 (Astek). Acid phosphatase isozymatic analysis was supplemented as needed according to the method of Hirata *et al.*, 1994. Chimera structure and tissue constitution were identified and classified according

to three layer origin (tunica-corpus) scheme of apical meristem (Schmidt, 1924). For the comparison of flowering dates among chimeras, revertant, control and other chimeral regenerates coming from the same tissue culture preparations were grown under the same conditions.

Pollen fertility by staining with acetocarmine, pod set (no. of pod-sets/total no. of crosses), and seed fertility (no. of fertile seeds/obtained seeds) were also investigated. Open pollinated and backcrossed seeds from chimeras were obtained to observe variation in the progeny.

3. Results and Discussion

One chimeral shoot from one graft out of 20 grafts was grown by *in vitro* approach-graft according to our established method (Noguchi *et al.*, 1992; Oguni *et al.*, 1996). Transplantation and propa-
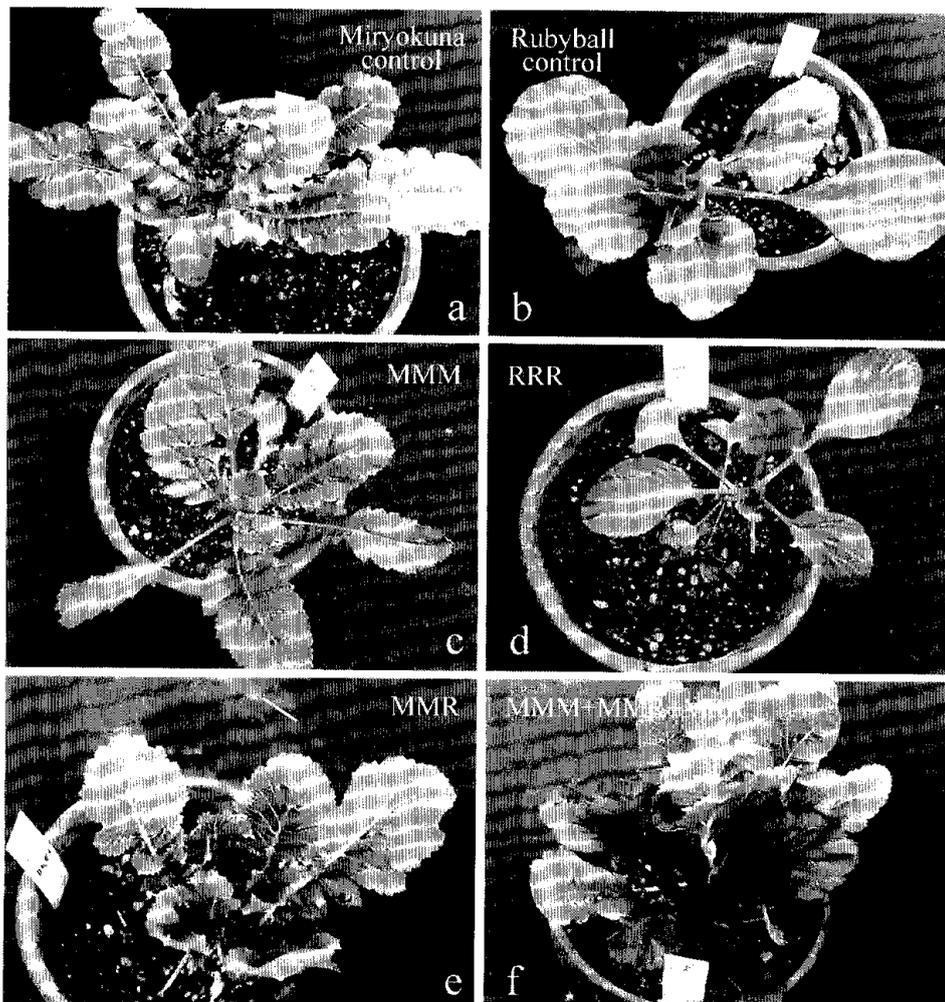
Fig. 1. Morphological characteristics of intergeneric chimeras between radish and cabbage.
a: Control radish cv. Miryokuna (*R. sativus*, cell layer constitution is described as MMM (L I-L II-L III)), **b:** Control cabbage cv. Ruby ball (*B. oleracea*, (RRR)),
c: Revertant to radish (MMM), **d:** Revertant to cabbage (RRR), **e:** Peripheral type (MMR) where anthocyanin pigmentation is observed along with leaf veins,
f: Mericlinal type (MMM+MMR+MRR) where MRR type is justified as anthocyanin pigmentation in leaf lamina without hair on the surface.
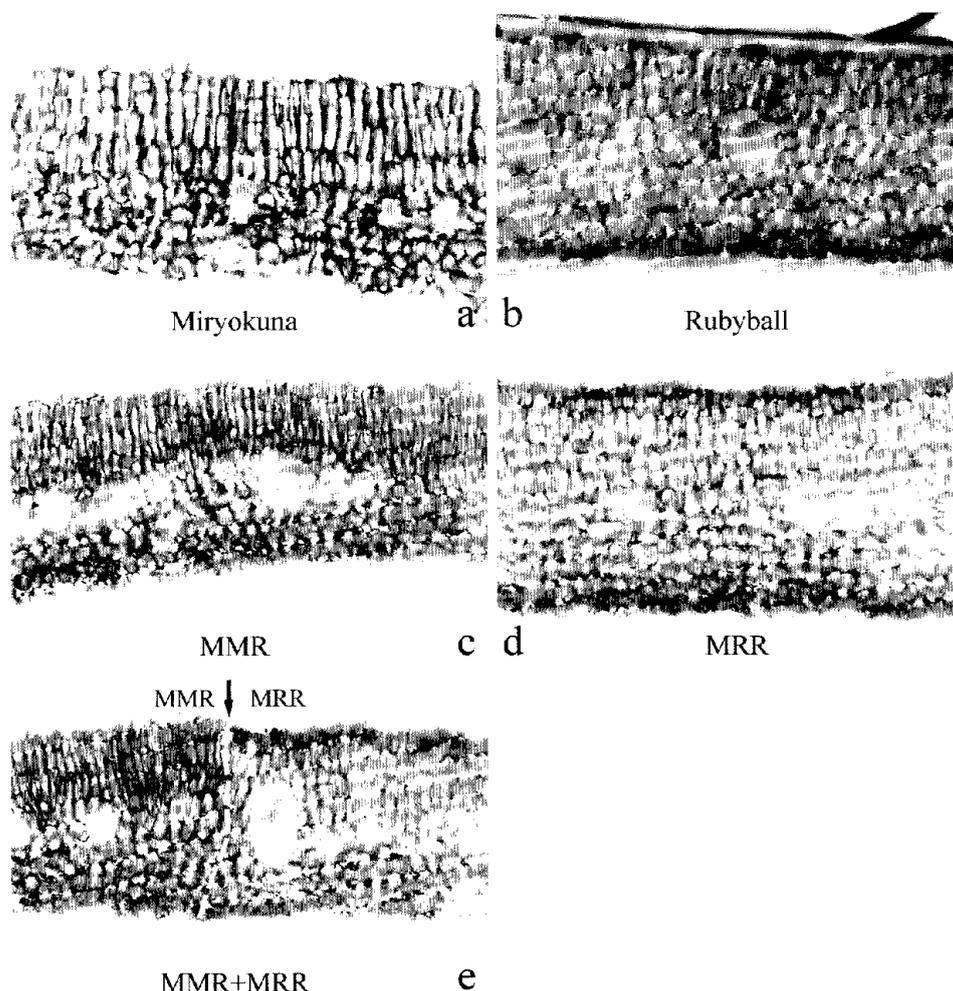
Fig. 2. Leaf tissue structure. **a:** Radish control which is characterized as three slender parenchyma cell layers. **b:** Control cabbage tissue is characterized as two or three relatively-cubical parenchyma cell layers. **c:** Peripheral MMR chimera which is characterized as slender parenchyma cell layers and irregular shape of vascular bundles. **d:** Peripheral MRR chimera which is characterized as anthocyanin pigmented sub-surface layer, relatively-cubical parenchyma cell layers and irregular shape of vascular bundles. **e:** Mericlinal chimera with MMR and MRR parts which are characterized as tissue parts from corresponding **c** and **d** part, respectively.

gation were performed from the chimeral tissues, resulting in 77 chimeras as the first generation of intergeneric chimeras. It is the first attempt to succeed to obtain intergeneric chimeras by *in vitro* graft-culture.

Type definition of 77 chimeras was done by morphological characteristics (**Fig. 1**), anthocyanin pigmentation pattern (L II and/or L III layer), leaf tissue structure by making the transversal sections (**Fig. 2**), surface layer (LI) structure by sump method (**Fig. 3**), and amplified DNA pattern by PCR analysis using *atp6* gene primer (radish specific marker is 1489bp fragment and cabbage marker is 925bp fragment in **Fig. 4**). These chimeral structures were classified (**Table 1**) according to three layers of origin of the apical meristem (Schmidt, 1924) as in the case of inter-

specific chimeras (Noguchi *et al.*, 1992). Results showed that more than half of the chimeras (43/77= 55.8%) were revertant to radish (LI-LII-LIII =MMM from the surface layer to the inner one), four (5.2%) were revertant to red cabbage, 14 (18.2%) were periclinal MMR type, only one (1.3%) was peripheral chimera MRR type, and the other 15 (19.5%) were more or less mericlinal chimeras of the mixed type with MMM, MMR and/or MRR. Mericlinal or mixed chimeras are described as MMM+MMR+MRR in **Fig. 1f**.

General characterization of those intergeneric chimeras could be identified based on the classification criteria of interspecific chimeras (Noguchi *et al.*, 1992). However, chimeral structures changed by the replacement of cell layer constitution along with developmental stages and seasons. The general
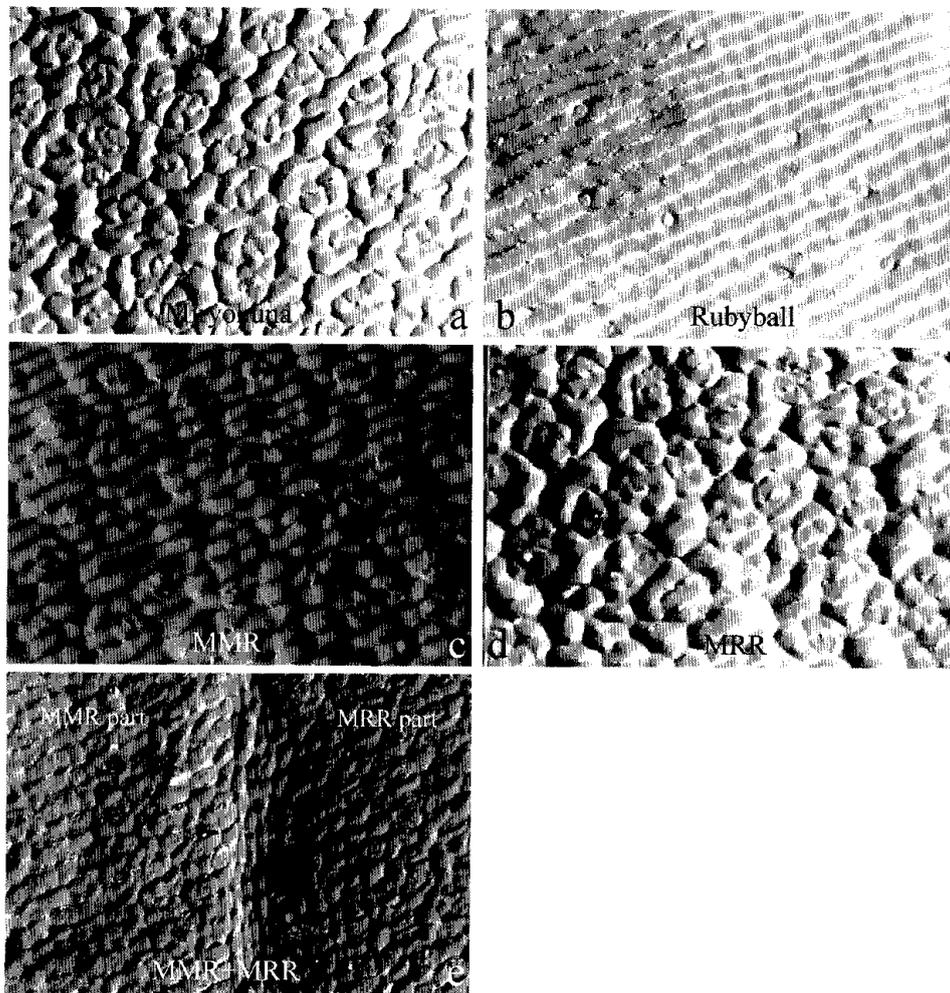
Fig. 3 Surface cell layer structure of leaf. The surface layer constitution is justified as a indicator of rolling condition by sump method accompanied by the existence of trichome in radish tissue and wax in cabbage one. **a:** Radish cv. Miryokuna control shows uneven surface. **b:** Cabbage cv. Ruby ball shows smooth surface with small guard cell traits. **c, d** and **e:** Surface structure of peripheral chimera MMR, MRR and mixed chimera MMR+MRR are the same as that of radish, reflecting cell layer construction.
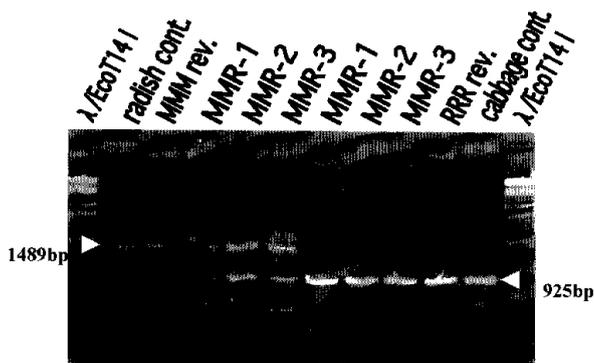
Fig. 4 Tissue layer constitution by PCR analysis. PCR band patterns generally reflect cell layer constitutions.

Amplified fragment patterns using *atp6* primers are shown from the left to the right, λ/Eco T14I, Miryokuna cont., revertant type, peripheral MMR type (lanes from MMR-1 to MMR-3), peripheral chimera MRR (lanes from MRR-1 to MRR-3), revertant RRR and Ruby ball cont.

developmental stages and seasons. The general patterns of layer replacement are listed in **Table 1**. Every layer displacement could occur in both directions from M to R and *vice verse*. During the summer season growth of cabbage seems to be predominant over that of radish. On the contrary, growth of radish seems to be dominant over that of cabbage in winter and cool seasons. A distinctive structure was discovered, that is a putative four-layer origin of apical meristem. In the revertant type of 43 chimeras, red cabbage emerged from the basal part of stem and root part of radish type of revertants in summer season (**Table 1** and **Fig. 5**). Morphological characteristics in the revertants were not different from those of other revertants nor the control. However, ten out of 43 radish-revertants were recognized to emerge of cabbage from inner tissue of the revertant type. If red cabbage tissue exists in the L Ⅲ layer of the leaf or in the stem,

Table 1. General characteristics of intergeneric chimera between *Raphanus* and *Brassica*

Chimera type	in the Spring				in the Summer	
	Cell layer constitution	PCR pattern	Petal color	Pollen fertility	Cell layer constitution	exchanged cell layer and direnction
Miryokunal (Cont)	MMM	M type	white	96.3%	MMM	no
Rudy ball (Cont)	RRR	R type	yellow	93.2%	RRR	no
Revertant	MMM	M type	white	96.1%	MMM	no
		M+R type	white		MMMR	4th layer express
	RRR	R type		—	RRR	no
Periclinal	MMR	—	white	92.1%	MMM+MMM, MMR+RRR	R→M (LIII), M→R (LI,II)
	MRR	M type	pale yellow	67.1%	MMM, MMM+MMR	R→M (LII, LIII)
Mericlinal	MMM+MMR		white	—	MMM+MRR	M→R (LII)
	MMM+MRR	—	white+pale yellow (chimeric)	—	MMM+RRR	M→R (LI)
	MMR+MRR	M+R type	white+pale yellow (chimeric), sectorial	86.5%	MMM+MMR+MRR, MRR	M→R (LII), R→M (LIII)
	MMM+MMR+MRR	M+R type	white+pale yellow (chimeric)	—	MMM, MMM+MRR, MMR+MRR, RRR	M→R (LI)

Fig. 5 Appearance of cabbage shoot from putative MMMR type which was justified as revertant radish type in an earlier stage and included cabbage tissue in the more inner L III layer.

anthocyanin pigmentation can be observed along the leaf veins (**Fig. 1e**) or base of petiole. However, anthocyanin pigmentation, morphological characteristics, acid phosphatase isozymatic specific band and radish specific band by *atp6* primer were not found. Hence, this type would be identified as the MMMR type, one type of four-layer origin. Four layer origin of apical tissue is also recognized in other interspecific chimera of *Brassica* (Motegi *et al.*, 1997) and in some cases of higher plants (Masubuchi, 1977). Recently, another probable four

- layered type was found, but very different from the type reported here (Xiao *et al.*, 1998).

Other results in which morphological identifications were different from PCR analyses were observed in MMR and MRR. This type of conflicted results was also observed in petal DNA by PCR and isozyme analysis of interspecific chimera between *B. oleracea* and *B. napus* (Motegi *et al.*, 1997). Although there exists this difficulty, we could have identified the more precise type of chimeras with many characteristics such as in **Table**
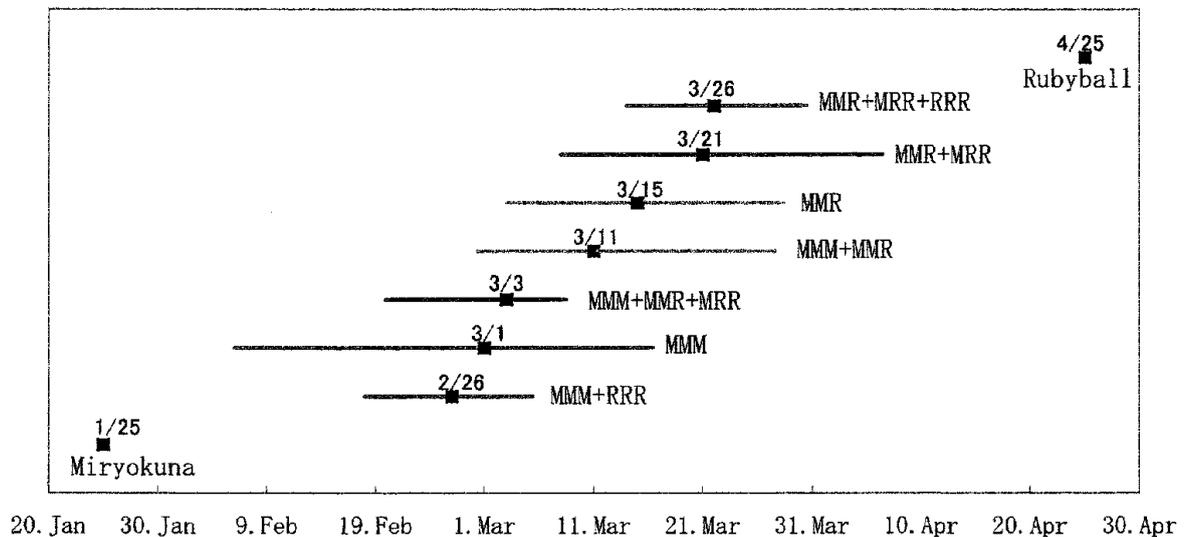
Fig. 6 Flowering date spectrum of intergeneric chimeras between cabbage and radish.

1. Sometimes tissue culture from the chimera is applicable to justify the inner tissue constitution in question and transgenic plants with GUS gene are also adopted as a parental material for chimera synthesis using a critical marker with one of two parents (Horiuch et al., 1994). Physiological interactions were clearly observed. For example, flowering dates were delayed in the chimeras which included cabbage tissue (**Table 1** and **Fig. 6**) as well as in those of interspecific chimeras between B. oleracea and B. campestris, and between B. oleracea and B. napus, because cv. Ruby ball requires strong vernalization for flowering (Noguchi et al., 1992; Noguchi and Hirata, 1994). In general, interspecific and intergeneric chimeras are sterile (Masubuchi, 1977). Pollen fertilities of the intergeneric chimeras were relatively high ranging from 67.1% in MRR to 96.1% in revertant radish compared with those in both parents (96.3% in radish and 93.2% in cabbage) (**Table 1**), but both pod set and seed set were not obtained (0%) in the chimeras which included Ruby ball tissue in the L II germinal cell layer except for the Ruby ball revertant (26.0% in pod set and 62.9% in seed set) in spite of crossing it with a Ruby ball control. Pollen fertility of the chimera where L II was occupied with Ruby ball was markedly lower than those of the other structures. Fertile seeds were produced at higher frequency in the progeny of L II (M) crossed with radish. However, variation has not yet been obtained in the progeny from the backcrosses with chimeras.

Acknowledgment

References

Gamborg, O.L., Miller, R.L., Ojima, K., 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res., **50**: 151-158.

Hirata, Y., Yagishita, N., Yamamoto, K., Sugimoto, M., 1992. Interspecific graft chimera formation between Brassica oleracea and B. campestris. Japan. J. Breed., **42**: 203-212.

Hirata, Y., Noguchi, T., Oguni, S., Kan, T., 1994. Genetic constitution of germ cells in intervarietal and interspecific chimeras of Brassica induced by in-vitro grafting. Theore. Appl. Genet., **89**: 249-254.

Hirata, Y., Noguchi, T., Kita, M., Kan, T., Ledoux, L., 1995. Graft transformation and its mechanism in higher plants. In: Oono, K. et al. (Eds.): Modification of Gene Expression and Non-Mendelian Inheritance 325-340. NIAR, Tsukuba.

Hirata, Y., Takeda, Y., Noguchi, T., Motegi, T., Kita, M., 1997. Proc. ISHS Symposium on Brassicas, 225-226 (Rennes, France).

Horiuchi, H., Hirata, Y., Noguchi, T., Takeda M., Ishiwata, K., 1994., Characteristics of floral organs in interspecific chimera between Brassica oleracea and transgenic B. napus. Japan. J. Breed., **44** (Suppl. 2) : 306.

Masubuchi, Y., 1977. Chimera and Grafting. In: Kihara, H. et al. (Eds.): Plant Genetics IV. Morphogenesis and Mutation, pp. 287-321. Shokabo, Tokyo (in Japanese).

Murashige, T., Skoog, F, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant., **15**: 473-497.

Motegi, T., Hirata, Y., Kita, M., Noguchi, T., Sugitani, C. , 1997. Intergeneric chimera formation between radish (Raphanus sativus) and cabbage (Brassica oleracea) and its characterization. Breed. Sci., **47** (Sup. 1): 507.

Noguchi, T., Hirata, Y., Yagishita, N., 1992. Intervarietal and interspecific chimera formation by in vitro graft-culture method in Brassica. Theore. Appl. Genet., **83**: 727-732.

Noguchi, T., Hirata, Y., 1994. Vegetative and floral charac-

teristics of interspecific *Brassica* chimeras by *in vitro* grafting. Euphytica, **73**: 273 – 280.

Oguni, S., Hirata, Y., Kan, T., Kita, M., Horiuchi, H., Noguchi, T., 1996. Chimera synthesis and propagation systems by *in vivo* and *in vitro* graft methods in *Brassica*. Acta Hort., **407**: 227 – 232.

Schmidt, A., 1924. Histologishe Studien an phanerogamen Vegetationspunkten. Bot. Arch., **8**: 345 – 404.

Takeda, Y., Hirata, Y., Motegi, T. , Kita, M., 1997. Analysis of mitochondrial DNA in the progenies derived from interspecific chimeras between *Brassica oleracea* and *B. campestris*. Breed. Sci., **47** (Suppl. 2): 347.

Taller, J., Hirata, Y., Yagishita, N., Kita, M., Ogata S., 1998. Graft – induced genetic changes and the inheritance of several characteristics in pepper (*Capsicum annuum* L.). Theore. Appl. Genet., **97**: 705 – 713.

Xiao, Q. B., Hirata, Y., Motegi, T., Noguchi, T., 1998. Morphogenesis and interaction between different cells and tissues in intergeneric chimeras between radish (*Raphanus sativus*) and cabbage (*Brassica oleracea*). Breed. Sci., **48** (Suppl., 2): 323.

Original Paper

The Functional Expression of the *CHS-D* and *CHS-E* Genes of the Common Morning Glory (*Ipomoea purpurea*) in *Escherichia coli* and Characterization of Their Gene Products.

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Abstract

The genes encoding chalcone synthase (CHS) in the common morning glory (*Ipomoea purpurea*) comprise a multigene family, and they are divided into two subfamilies. The genes in a subfamily including the *CHS-A, CHS-B* and *CHS-C* genes are distantly related to the other known *CHS* sequences in a phylogenetic tree, whereas the *CHS-D* and *CHS-E* genes in another subfamily are more closely related to the well-characterized *CHS* genes.

As an initial step to elucidate biological function of these *CHS* genes in *I. purpurea*, the *CHS-D* and *CHS-E* cDNAs were expressed in *Escherichia coli* with different expression systems. The recombinant CHS-D and CHS-E proteins both showed CHS activity to produce naringenin chalcone.These results are discussed with regard to the biological roles of the *CHS-D* and *CHS-E* genes in flower pigmentation in *I. purpurea*. We have also discussed these CHS-D and CHS-E enzyme as members of plant specific polyketide synthases.

Key words chalcone synthase, *Ipomoea purpurea*, *p*-coumaroylacetoacetic acid synthase, bisnory-angonin

Introduction

Flavonoids play important roles in flower pigmentation and in many other biological functions, including protection against UV light, plant-microbe interactions, and male fertility (Harborne, 1994; Shirley, 1996). Chalcone synthase (CHS) catalyzes a successive condensation of three molecules of malonyl-CoA and one molecule of *p*-coumaroyl-CoA to form naringenin chalcone. The CHS enzyme has been regarded to be a key enzyme for flavonoid biosynthesis and is now considered to be a members of plant-specific polyketides synthases (PKSs) (Schröder, 1997), which catalyzes the linking of acyl-CoA subunits by repetitive decarboxylative condensations (Hopwood, 1997). The *CHS* genes comprise a multigene family in many species, although a few plants such as *Antirrhinum* and *Arabidopsis* are known to carry a single copy of the gene (Harborne, 1994). Durbin *et al.* (1995) have isolated four *CHS* genes, *CHS-A, CHS-B* and *CHS-C* and a pseudogene, *CHS-PS*, from *Ipomoea purpurea* and found that they are members of the *Ipomoea CHS* gene family, which is comprised of 13 *CHS* genes from seven *Ipomoea* species. These *Ipomoea CHS* genes are distantly related to the other known *CHS* sequences in a phylogenetic tree.

Another subfamily of the *CHS* genes, *CHS-D* and *CHS-E*, were later isolated from pigmented flower buds of *I. purpurea* (Fukada-Tanaka *et al.*, 1997; Habu *et al.*, 1997). The nucleotide and deduced amino acid sequence of the *CHS-D* showed 79.9 and 85.9% identities to those of *CHS-E*, respectively. The *CHS-D* and *CHS-E* genes do not belong to the previously characterized *Ipomoea* CHS gene subfamily and are more closely related to the *CHS* sequences found in other plant species.

These *CHS* genes in the newly identified *CHS* gene subfamily are expressed predominantly in the pigmented buds, and the *CHS-D* gene is responsible for pigmentation in flower limbs and stems while the *CHS-E* gene is expressed mainly in faintly pigmented flower tubes (Fukada-Tanaka, *et al.*, 1997; Johzuka-Hisatomi *et al.*, 1999; Durbin *et al.*, 2000). Indeed, the mutable *flaked* plant having the transposable element *Tip100* inserted into the *CHS-D* gene produces white flowers with colored spots and sectors as well as green stems with red sectors (Hisatomi *et al.*, 1997; Habu *et al.*, 1998). These variegated phenotypes in flowers and stems are caused by recurrent somatic mutations due to excision of Tip100 from the *CHS-D* genes. Although these lines of circumstantial evidence were consistent with the notion that the *CHS-D* gene indeed encodes the CHS enzyme in anthocyanin biosynthesis pathway which forms naringenin chalcone, no substantial biochemical data concerning enzyme activities of these *CHS* gene products are available. It would be interesting to ask whether all the *CHS* genes belonging to the different subfamily of *I. purpurea* equally retain the enzyme activity of the CHS.

As an initial step to elucidate biological function of these *CHS* genes in *I. purpurea*, we have chosen to characterize the *CHS-D* and *CHS-E* genes, since not only the mode of expression of there genes but also their genomic structures are well documented (Johzuka-Hisatomi *et al.*, 1999; Durbin *et al.*, 2000). We have attempted to clarify the function of them by characterizing the products of the enzymatic reactions that are catalyzed by the recombinant proteins yielded by the functional expression of their cDNAs in *E. coli* K12 derivatives. In this study, we showed that both CHS-D and CHS-E gene products indeed carry the CHS activity to produce naringenin chalcone.

2. Materials and Methods

2.1 Bacterial expression systems used

The full length of cDNAs of the *CHS-D* and *CHS-E* genes were described previously (Fukada-Tanaka *et al.*, 1997; Habu *et al.*, 1997). For the preparation of the recombinant chalcone synthases, we used the bacterial expression systems; the pET system (pET22b(+) in *E. coli* (BL21(DE3)pLysS; Novagen; pRSET C in *E. coli* (BL21(DE3)pLysS; Invitrogen) and the IMPACT system (pCYB3 and pTYB3 in *E. coli* ER2267 and ER2566; New England Biolabs). In the pET system, six histidine residues were added to the C- or N-termini of CHS-D. IMPACT systems are exploited for obtaining

intact recombinant proteins using a self-cleavable affinity tag derived from a protein splicing element (Chong *et al.*, 1997).

(1) Construction of plasmid pSIO11 producing intact-CHS-D proteins

To introduce *Nco* I and *Sap* I sites at the N- and C-termini of the *CHS-D* ORF, respectively, the primers (Primer 1) 5'-CGAAG<u>CCATGG</u>TGACC-GTCG-3' and (Primer 2) 5'-TTT<u>GGCTCTTCC</u>-GCATGCTGGGACGCTATG-3' were used for the PCR amplification. (**Fig. 1A**) The PCR reaction with 10 pmol each of these primers with 1 ng of *CHS-D* cDNA was carried out for 30 cycles consisting of denaturation at 93 ℃ for 40 s, annealing at 42 ℃ for 40 s and extension at 72 ℃ for 60 s with 2.5 U of ExTaq DNA polymerase (Takara). The obtained PCR products were digested with *Nco* I and *Sap* I and ligated into *Nco* I and *Sap* I sites of pCYB3 to yield pSIO3. To express the CHS-D gene driven by T-7 promoter, the *Nco* I and *Kpn* I fragment containing the *CHS-D* ORF of pSIO3 was then cloned into the *Nco* I and *Kpn* I sites of pTYB3 to produce pSIO11. The expression plasmid pSIO11 was introduced into *E. coli* ER2566.

(2) Construction of plasmid pSIO33 for CHS-D-His protein and pSIO57 for His-CHS-D protein

To construct pSIO33 which can produce the CHS-D derivative with tagged six histidine-residues at its C-terminus (CHS-D-His), the primer (Primer 3) 5'-TAATACGACTCACTATAGGG-3' containing the T7 promoter sequence and the primer (Primer 4) 5'-TG<u>GCGGCCGC</u>TGCTGGGACGCTATG-3' with a *Not* I site were used for amplification of the DNA fragment carrying the *CHS-D* ORF. (**Fig. 1B**) The PCR amplification with 10 pmol each of these primers together with 1 ng of pSIO11 containing the *CHS-D* cDNA sequence was carried out for 30 cycles consisting of denaturation at 93 ℃ for 40 s, annealing at 52 ℃ for 40 s and extension at 72 ℃ for 60 s with 2.5 U of ExTaq DNA polymerase. The obtained PCR products were cleaved with *Xba* I and *Not* I and cloned into the *Xba* I and *Not* I sites of pET22b(+) to yield the plasmid pSIO33.

To obtain pSIO57 producing the CHS-D derivative with tagged six histidine-residues at its N-terminus (His-CHS-D), the primers (Primer 5) 5'-<u>GCTAGC</u>ATGGTGACCGTCGAGGAGGTCA-3' with an *Nhe* I site (Primer 6) 5'-<u>CCATGG</u>TTA-TGCTGGGACGCTATGGAG-3' with an *Nco* I site were employed, and the PCR amplification with 10 pmol each of these primers together with 1 ng of pSIO11 was performed for 30 cycles consisting of denaturation at 93 ℃ for 40 s, annealing at 56 ℃ for 40 s and extension at 72 ℃ for with 2.5 U of ExTaq DNA polymerase. (**Fig. 1C**) The resultant PCR
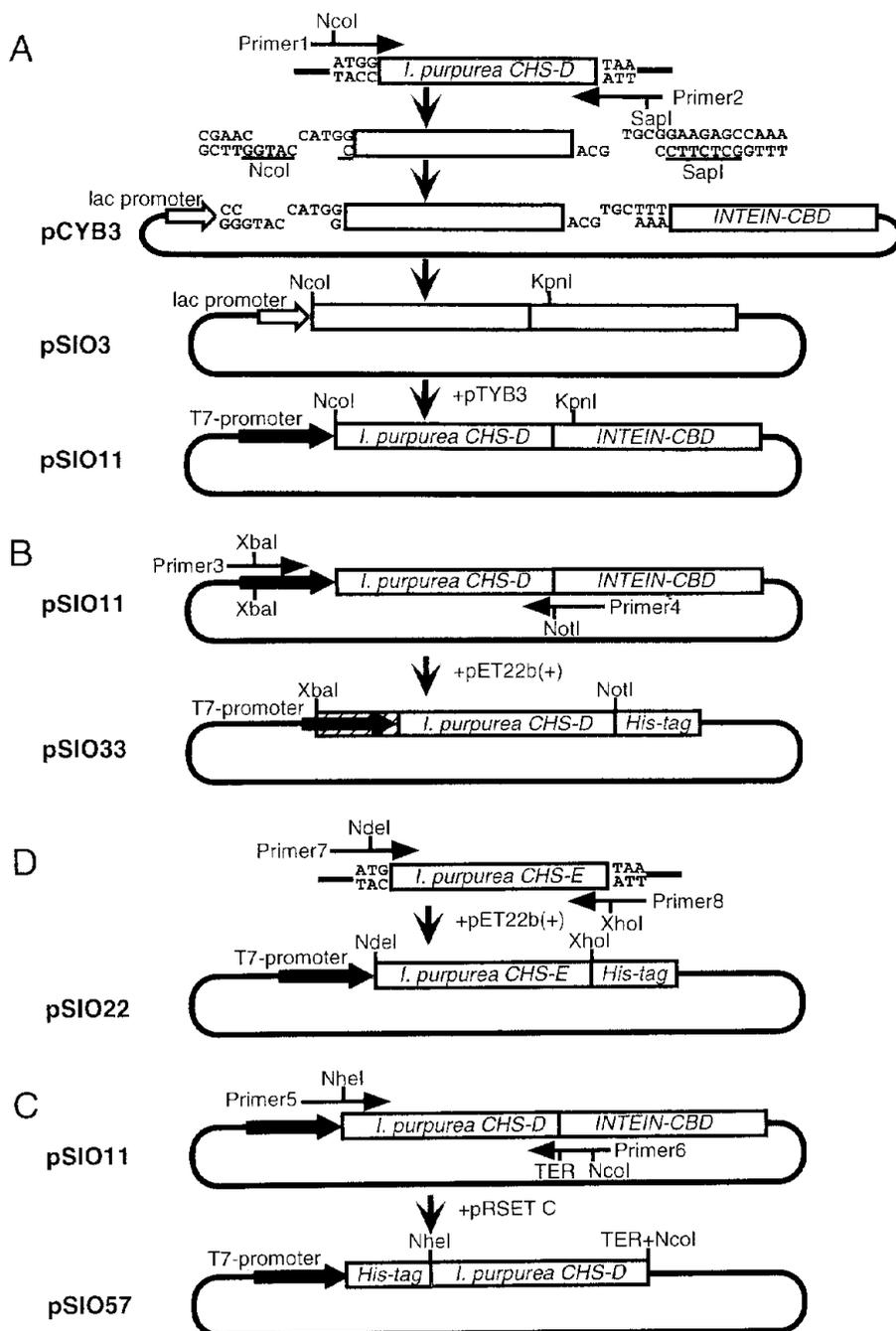
Fig. 1A-D. Prasmid constructs for the heterologous expression of chalcone synthases in *E. coli*. Only the relevalent restriction sites are indicated.TER; stop codon. See Materials and Methods.

products were digested with *Nhe* I and *Nco* I and ligated into the *Nhe* I and *Nco* I sites of pRSET C to yield the plasmid pSIO57. These pSIO33 and pSIO57 plasmids were introduced into *E. coli* BL21(DE3)pLysS for production of recombinant CHS-D-His and His-CHS-D proteins, respectively.

(3) *Construction of plasmid pSIO22 producing CHS-E-His proteins*

For obtaining the CHS-E derivative with tagged six histidine-residues at its C-terminus (CHS-E-His), the primers (Primer 7) 5'-CAGCTAC<u>CATATG</u> GTGACCG-3' with a *Nde* I site and (Primer 8) 5'-

TTGGC<u>CTCGAG</u>AGCTGAAACACTATG-3' with a *Xho* I site were used to amplify the fragment containing the *CHS-E* ORF. (**Fig. 1D**) The PCR amplification with 10 pmol each of these primer together with 1 ng of the *CHS-E* cDNA was carried out for 30 cycles consisting of denaturation at 93 °C for 40s, annealing at 56 °C for 40 s and extension at 72 °C for 60 s with 2.5 U of ExTaq DNA polymerase. The resultant PCR products were cleaved with *Nde* I and *Xho* I and ligated into the *Nde* I and *Xho* I sites of pET22b(+) to construct the plasmid pSIO22, which was introduced into *E. coli* BL21 (DE3)pLysS for production of recombinant CHS-E

-His proteins.

2.2 Purification of the expression proteins

The following buffers were used for purification of the expression proteins: Buffer I (4 mM imidazole, 40 mM K_2HPO_4, 0.1M NaCl, 0.002% RNase, 0.001% DNase, 0.2 mM phenylmethanesulfonyl fluoride (PMSF), 0.5% polyoxyethylene (10) octylphenyl ether (Triton X-100), pH 7.9), Buffer II (40 mM imidazole, 20 mM K_2HPO_4, 0.5M NaCl, pH 7.9), Buffer III (0.5M imidazole, 15 mM K_2HPO_4, 10% glycerol, pH 7.5), Buffer IV (20 mM K_2HPO_4, 0.5M NaCl, 0.1 mM EDTA, 0.002% RNase, 0.001% DNase, 0.2 mM PMSF, 0.5% Triton X-100, pH 8.0), Buffer V (50 mMβ-mercaptoethanol (2-ME), 20 mM K_2HPO_4, 50 mM NaCl, 0.1 mM EDTA, pH 8.0), Buffer VI (20 mM K_2HPO_4, 50 mM NaCl, and 0.1 mM EDTA, pH 8.0), Buffer VII (100 mM K_2HPO_4, 1 mM EDTA, 10% glycerol, pH 8.0).

We purified hexahistidyl-tagged proteins, CHS-D-His, His-CHS-D and CHS-E-His, from *E. coli* BL21(DE3)pLysS containing the pET vector plasmids, pSIO33, pSIO57 and pSIO22, respectively. These *E. coli* strains were grown in 10 m*l* of LB medium containing ampicillin (100 μg m*l*$^{-1}$) and chloramphenicol (25 μg m*l*$^{-1}$) at 37 ℃ to the saturation. The cells were then transferred to one liter of LB medium containing ampicillin (100 μg m*l*$^{-1}$) at 37℃ for 3 hours. After cultures were cooled down to 25 ℃, isopropyl-β-D-thiogalactopyranoside(IPTG) was added to the final concentration of 1 mM to induce for protein expression, and cultures were further incubated at 25 ℃ for 3 hours. The cells were collected by centrifugation, resuspended in 10 m*l* Buffer I, and lysed by intermittently modulated sonication for 20 min. on ice. The lysate was centrifuged at 10000 x g for 30 min. at 4 ℃ , and then, the supernatant was loaded on to 2 m*l* PROBOND Resin (Invitrogen). The resin was washed with 500 m*l* Buffer II. The bound protein was eluted with 10 m*l* Buffer III.

The *E. coli* strain ER2566 harboring pSIO11 was cultured in 10 m*l* of LB medium containing ampicillin (100 μg m*l*$^{-1}$) and tetracycline (25 μg m*l*$^{-1}$) and incubated at 37 ℃ until full-growth culture to obtain INTEIN-CBD tagged protein. The cultures were transferred in one liter of LB medium containing ampicillin (100 μg m*l*$^{-1}$) and incubated at 37 ℃ for 3 hours. After cultures were cooled down to 25 ℃ , 1 mM IPTG was added to induce for protein expression, and cultures were further incubated at 25 ℃ for 5 hours. The cells were harvested and resuspended in 10 m*l* Buffer IV, and lysed by intermittently modulated sonication for 20 min on ice. The lysate was centrifuged at 10000 x g for 30

min. at 4 ℃, and the supernatant was loaded on to 2 ml Chitin Beads (NEB). The Chitin Beads column washed with one liter of Buffer IV without RNase, DNase and phenylmethylsulfonylfluoride (PMSF). For on-column cleavage reaction (Chong *et al.*, 1997), we were flushed the column with 15 m*l* Buffer V to uniformly distribute 2-ME throughout the column. The column was left at 4 ℃ overnight to release intact-CHS D-His protein and the resultant protein was eluted using 10 m*l* Buffer VI.

The fractions from the affinity columns that contain CHS-D-His, His-CHS-D and CHS-E-His proteins in Buffer III and the intact-CHS-D protein in Buffer IV were concentrated to about 500 μl using CENTRIPREP10 (Amicon) at 4000 x g at 4 ℃. For concomitant desalting, replacement and concentration, 10 m*l* Buffer VII were added to the protein solutions and concentrated using CENTRI-PREP10 at 4000 x g at 4 ℃ twice. These manipulations restored the enzyme activity. The protein concentrations were measured by Bio-Rad Protein Assay (Bio-Rad Laboratories)

2.3 Assays of the CHS activities

The standard incubation (300 μl) mixture contains 50 nmol p-coumaroyl-CoA (starter CoA-ester), 150 nmol malonyl-CoA and 0.2 nmol protein in the Buffer VII . The reaction was stopped after 2 hours at 30 ℃ by adding 20% HCl (30 μl) to convert the yielded chalcone into isomeric flavanone. The products were extracted with ethyl acetate (EtOAc) (330 μl x 2), and EtOAc layer was washed with distilled water (500 μl) and concentrated by air blowing. The residue was diluted with 20 μl of MeOH (0.1% TFA), and subjected to HPLC (JASCO 880, JASCO) using TSK-gel ODS-80Ts (4.6 x 15 cm, Tosoh) monitoring by a multichannel UV detector (MULTI 340, JASCO) at 290 nm and 360 nm with a flow rate of 0.8 m*l* min^{-1}. A UV spectrum (198-400 nm) was recorded every 0.4 s. The solvents used for gradient elution were solvent A (MeOH, 0.1% TFA) and solvent B (Water, 0.1% TFA). The gradient conditions are as follows: 0-5 min, 30% solvent A in solvent B; 5-17 min, liner gradient to 60% solvent A in solvent B; 17-30 min, 60% solvent A in solvent B. Authentic naringenin gave a peak at retention time of 23.0 min under these conditions. The standard calibration curves were made from solutions of a known concentration of authentic sample. The pET22b (+) expression vector without the CHS sequence was used as a negative control.

2.4 On-line HPLC-ESI MS analysis

A Hewlett-Packard HPLC 1100 series (Wil-

mington, DE) coupled to a Finnigan MAT LCQ ion trap mass spectrometer (San Jose, CA) fitted with an ESI source was used. The separation was carried out on a TSK-gel ODS-80Ts (4.6 x 15 cm, Tosoh) with 45% MeOH in water containing 0.1% TFA as mobile phase. The flow rate of the mobile phase was 0.8 ml min^{-1}. The ESI capillary temperature and capillary voltage were 275 ℃ and 3.0 V, respectively. The tube lens offset was set at 20.0 V. All spectra were obtained in the positive mode; over a mass range of m/z 150-500, at a range of one scan every 2 s. The collision gas was helium, and the relative collision energy scale was set at 30.0% (1.5 eV).

3. Results

3.1 The functional expression of CHS-D and CHS-E genes and purification of their gene products from E. coli.

The plasmid pSIO11 to express INTEIN-CBD tagged CHS-D protein was constructed and was expressed in the *E. coli* strain ER2566. The cell free extract that was harvested after induction of IPTG for 5 hours was used to obtain the natural CHS-D enzyme (intact-CHS-D) in sequence by on-column cleavage reaction. The recovery of active enzyme after the cleavage reaction by β-mercaptoethanol (2-ME) was about only 70 μg per one liter culture. However, the enzyme activity as chalcone synthase was retained after the on column reaction using thiol reagent. The low yield of the expressed recombinant protein using the above IMPACT system tempted us to construct an alternate expression system. We chose to synthesize these enzymes with hexahistidyl-terminal extensions to facilitate their purification. For that purpose, we constructed pSIO33 producing CHS-D-His (six histidine residue at the C-terminus) and pSIO57 producing His-CHS-D (six histidine residue at the N-terminus) to express them with the pET system. Both His-tagged constructs produced the recombinant proteins that are readily bound to the Ni^{2+} affinity column. The yields of the purified enzymes were about 2-3 mg per one liter culture. The CHS-E-His (C-terminal His-tagged) protein was expressed in the pET system and the yield of the purified enzyme after the Ni^{2+} affinity column (PROBOND Resin) purification step was almost the same as those of CHS-D-His and His-CHS-D.

3.2 Enzyme activities of the CHS-D and CHS-E proteins

The enzyme activities of three different recombinant CHS-D protein derivatives, intact-CHS-D,

CHS-D-His (C-terminal His-tagged) and His-CHS-D (N-terminal His-tagged), which were expressed in *E. coli*, were examined. The results of the HPLC analyses of the products are shown in the **Fig. 2**. Reaction product profiles of all three enzymes were almost identical. The first major peak at Rt=17.9 was identified as p-coumaroyltriacetic acid lactone (CTAL), because it gave a molecular ion at m/z 273 [M+H]$^+$ (LC-MS) and a fragment at m/z 227(MS/MS). The peak afforded the UV spectrum showing an absorption maximum at 332 nm that is in accord with this structure. The second major peak at Rt=23.0 was identified as naringenin, because it gave also a molecular ion at m/z 273 [M+H]$^+$ (LC-MS) as well as the typical fragment of flavanone at m/z 153(MS/MS). The structure was further confirmed by the UV spectrum with an absorption maximum at 290 nm. Two small peaks between these two major peaks are inferred to be *trans*- and *cis*-isomers of bisnoryangonin, judging from these mass numbers, [M+H]$^+$ at m/z 231 and the characteristic UV absorption at 365 nm (Akiyama, 1998). The above results reveal that all those proteins (intact-CHS-D, CHS-D-His, His-CHS-D) catalyzed naringenin chalcone formation with malonyl-CoA and p-coumaroyl-CoA as substrates at the specific activity of 68, 56, 49 μKat kg^{-1}, respectively. The intact-CHS-D protein that is obtained by cleavage reaction using 2-ME; one of the typical thiol reagents showed the same or somewhat better specific activity for naringenin chalcone than those of His-tagged recombinant protein. The on column cleavage reaction with 30-50 mM of dithiothreitol or 100 mM of hydoxylamine gave the same results (data not shown). It is well known that the chalcone synthase releases bisnoryangonin in much higher relative rate to naringenin when the reaction mixture contains various thiol reagents (Kreuzaler *et al.*, 1975). Our results show that the enzyme recovers its genuine activity when it is assayed free from the thiol reagents. Though the enzyme has once contacted with the thiol reagents. Thus, we can conclude that all those recombinant CHS-D proteins showed the almost same enzyme activities regardless of the intact form or the His-tagged forms.

For the *CHS-E* gene product, we have examined only the recombinant CHS-E-His derivative produced in *E. coli*. As is the case for the CHS-D enzymes, two major products, CTAL and naringenin were detected under the same assay condition (**Fig. 1**). Interestingly, the specific activity of the CHS-E-His proteins was found to be 158 μKat kg^{-1}, which is about three times higher than that of the corresponding CHS-D-His proteins. It is clear that the *CHS-E* gene also encodes chalcone synthase.
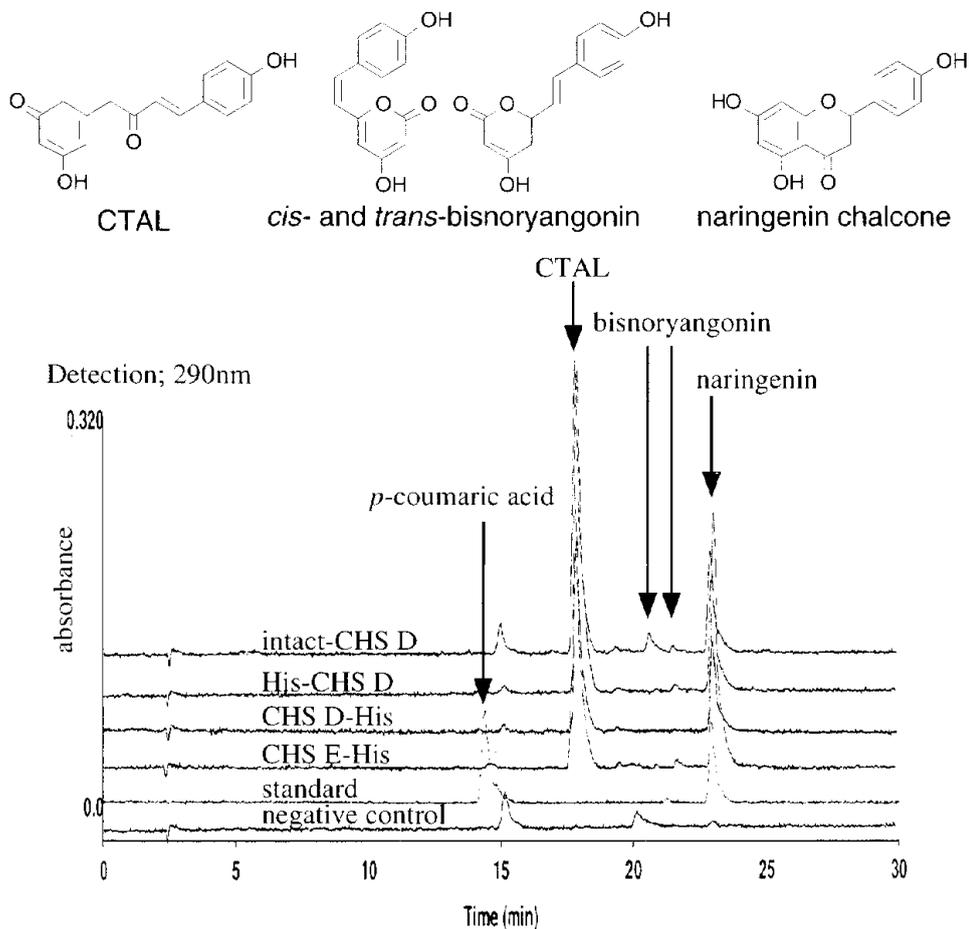
Fig. 2. HPLC elution profiles obtained for assays of heterologously expressed chalcone synthase activities. For assay and HPLC conditions, see Materials and Methods.

4. Discussion

4.1 The CHS-D and CHS-E genes and flower pigmentation

The CHS genes in the common morning glory as well as the Japanese morning glory (*Ipomoea nil*) comprise a multigene family (Durbin *et al.*, 1995; Fukada-Tanaka *et al.*, 1997). Among these CHS genes, the CHS-D gene is the most abundantly expressed in the pigmented flower buds and majorities of the remaining CHS mRNAs are produced from the CHS-E gene. The CHS-D and CHS-E genes are expressed predominantly in flower limbs and tubes, respectively (Fukada-Tanaka *et al.*, 1997; Johzuka-Hisatomi *et al.*, 1999; Durbin *et al.*, 2000). Moreover, the mutable *flaked* plants of *I. purpurea* having the transposable element *Tip100* inserted into the CHS-D gene produces white flower limbs with colored spots, and the CHS-D gene is scarcely expressed in the young flower buds of the mutable lines (Habu *et al.*, 1997; 1998). However, the flower tubes of these mutants are often lightly pigmented (Habu *et al.*, 1998; Johzuka-Hisatomi *et al.*, 1999). The observation presented here that both

CHS-D and CHS-E genes encode CHS enzymes further confirmed the previous notion that the function of the CHS-D and CHS-E gene products of *I. purpurea* is the chalcone synthase which is a key enzyme for anthocyanin biosynthesis. Although the specific activity of the CHS-E enzyme seems to be higher than that of the CHS-D protein, the pigmentation in flower tubes is much weaker than that in flower limbs. Probably, this apparent contradiction can be partially explained by the fact that the expression of the CHS-D gene is much more abundant than that of the CHS-E gene (Fukada-Tanaka *et al.*, 1997; Johzuka-Hisatomi *et al.*, 1999; Durbin *et al.*, 2000). Alternatively, one of the other structural genes in the anthocyanin biosynthesis pathway may be much scarcely expressed in flower tubes than in flower limbs.

Since the genomic as well as the cDNA sequences of the corresponding CHS genes between *I. purpurea* and *I. nil* are highly homologous each other (Johzuka-Hisatomi *et al.*, 1999), it is highly likely that both CHS-D and CHS-E genes of *I. nil* also encode CHS enzymes in anthocyanin biosynthesis pathway. Indeed, we are able to identify that a mutation conferring white flowers in *I. nil* is caused

by insertion of a transposable element into the *CHS -D* gene (A. Hoshino and S. Iida, unpublished results).

4.2 The CHS-D and CHS-E gene products and polyketide synthases

As **Fig. 2** shows, the CHS-D and CHS-E proteins produced CTAL, a well-known by-product of CHS, which was released from the enzyme prematurely in non-physiologically condition. Recently, a new type of the *CHS* gene, termed *HmS* for *p*-coumaroyltriacetic acid synthase, was isolated from *Hydrangea macrophylla* var. *thunbergii*, and its genuine product was found to be *p*-coumaroyltriacetic acid (Akiyama *et al.*, 1999). The sequences of the *CHS-D* and *CHS-E* genes showed higher similarity to that of the *Hms* gene than those of the *CHS-A* and *CHS-B* genes among the two subfamilies of the *CHS* genes, although *Ipomoea* and *Hydrangea* are distantly classified species.

Several plant polyketide synthases that utilized the compound other than phenylpropanoids were reported recently. For example, pyrone synthase, GCHS2, from *Gerbera hybrida* (Asteraceae) (Eckermann *et al.*, 1998; Zuurbier *et al.*, 1998) utilizes acetyl-CoA as its starter substrate, and bibenzyl synthase (BBS) cloned from *Phalaenopsis* sp. utilizes *m*-hydroxyphenyl-CoA and propionyl-CoA as the starters (Preisig-Müller *et al.*, 1995). Acridone synthase (ACS) cloned from *Ruta graveolens* was shown to utilize *N*-methylanthraniloyl-CoA as the starter substances (Junghanns *et al.*, 1995). As mentioned above, the *HmS* gene product from *Hydrangea macrophylla* has been identified to be a linear tetraketide synthase for the first time (Akiyama *et al.*, 1999). In every case, the constituents that are regarded to be derived from the products catalyzed by these polyketide synthases have been isolated from their mother plants, e.g. pyrone glycosides from *Gerbera hybrida* and secologanin derivatives from *Hydrangea macrophylla*.

Both Japanese and common morning glories carry at least five *CHS* genes which can be divided into two subfamilies (Durbin *et al.*, 1995; Fukada-Tanaka *et al.*, 1997). The first subfamily, represented by the *CHS-A* and *CHS-B* genes, are distantly related to the other known *CHS* sequences in a phylogenetic tree, whereas the other subfamily consisting of the *CHS-D* and *CHS-E* genes are more closely related to the well-characterized *CHS* genes. Although these *CHS* genes comprise two exons with an identical intron position, the introns of the *CHS-D* and *CHS-E* genes expressed abundantly in the pigmented buds are much longer than those of other *CHS* genes (Durbin *et al.*, 1995;

Johzuka-Hisatomi *et al.*, 1999). We have shown here that the *CHS-D* and *CHS-E* genes in *I. purpurea* indeed encode the chalcone synthases producing naringenin chalcone. It would be extremely interesting to ask whether the *CHS-A* and *CHS-B* gene products bear new polyketide synthase activities or they are also the ordinary CHS enzymes. We are currently characterizing the activities of the CHS-A and CHS-B proteins.

Acknowledgment

References

Akiyama, T., Shibuya, M., Liu, H.M., Ebizuka, Y., 1999. *p*-Coumaroyltriacetic acid synthase, a new homologue of chalcone synthase, from *Hydrangea macrophylla* var. thunbergii. Eur. J. Biochem., **263**: 834-839.

Akiyama, T., Doctor thesis, University of Tokyo, 1998.

Chong, S., Mersha, F.B., Comb, D.G., Scott, M. E., Landry, D., Vence, L.M., Perler, F.B., Benner, J., Kucera, R.B., Hirvonen, C.A., Pelletier, J.J., Paulus, H., Xu, M.Q., 1997. Single-column purification of free recombinant proteins using a self-cleavable affinity tag derived from a protein splicing element. Gene., **192**: 271-281

Durbin, M.L., Learn, G.H., Jr., Huttley, G.A. Clegg, M.T., 1995. Evolution of the chalcone synthase gene family in the genus *Ipomoea*. Proc. Natl. Acad. Sci. USA, **92**: 3338-3342.

Durbin, M.L., McCaig, B., Clegg, M.T., 2000. Molecular evolution of the chalcone synthase multigene family in the morning glory genome. Plant Mol. Biol., (in press).

Eckermann, S., Schröder, G., Schmidt, J., Strack, D., Edrada, R.A., Helariutta, Y., Elomaa, P., Kotilainen, M., Kilpeläinen, I., Proksch, P., Teeri, T.H., Schröder, J., 1998. New pathway to polyketides in plants. Nature., **396**: 387-390.

Fukada-Tanaka, S., Hoshino, A., Hisatomi, Y., Habu, Y., Hasebe, M., Iida, S., 1997. Identification of new chalcone synthase genes for flower pigmentation in the Japanese and common morning glories. Plant Cell Physiol., **38**: 754-758.

Habu, Y., Fukada-Tanaka, S., Hisatomi, Y., and Iida, S., 1997. Amplified restriction fragment length polymorphism-based mRNA fingerprinting using a single restriction enzy,me that recognizes a 4-bp sequence. Biochem. Biophys. Res. Commun., **234**: 516-521.

Habu, Y., Hisatomi, Y., Iida, S., 1998. Molecular characterization of the mutable *flaked* allele for flower varie-

gation in the common morning glory. Plant J., **16**: 371–376.

Harborne, J.B., 1994. The Flavonoids: Advances in Research since 1986. Chapmann and Hall, London., 499–535.

Hisatomi, Y., Yoneda, Y., Kasahara, K., Inagaki, Y., Iida, I., 1997. DNA rearrangements at the region of the dihydroflavonol 4–reductase gene for flower pigmentation and incomplete dominance in morning glory carrying the mutable *flaked* mutation. Theor. Appl. Genet., **95**: 509–515.

Hopwood, D.A., 1997 Genetic contributions to understanding polyketide synthesis. Chem. Rev., **97**: 2465–2497

Johzuka–Hisatomi, Y., Hoshino, A., Mori, T., Habu Y., Iida, S., 1999. Characterization of the chalcone synthase genes expressed in flowers of the common and Japanese morning glories. Genes Genet. Syst., **74**: 141–147.

Junghanns, K.T., Kneusel, R.E. Baumert, A., Maier, W., Gröger, D., Matern, U., 1995. Molecular cloning and heterologous expression of acridone synthase from elicited *Ruta graveolens* L. cell suspension cultures.

Plant Mol. Biol., **27**: 681–692.

Kreuzaler, F., Hahlbrock, K., 1975. Enzymatic synthesis of aromatic compounds in higher plants. Formation of bis–noryangonin (4–hydroxy–6[4–hydroxystyryl]2–pyrone) from p–coumaroyl–CoA and malonyl–CoA. Arch. Biochem. Biophys., **169**: 84–90

Preisig–Müller, R., Gnau, P. Kindl, H., 1995. The inducible 9, 10–dihydrophenanthrene pathway: characterization and expression of bibenzyl synthase and S–adenosylhomocysteine hydrolase. Arch. Biochem. Biophys., **317**: 201–207.

Schröder, J., 1997. A family of plant–specific polyketide synthase: facts and predictions. Trends Plant Sci., **2**: 373–378.

Shirley, B.W., 1996. Flavonoid biosynthesis: 'new' functions for an 'old' pathway. Trends plant Sci., **1**: 377–382.

Zuurbier, K.W.M., Leser, J., Berger, T., Hofte, A.J.P., Schröder, G., Verpoorte, R., Schröder, J., 1998. 4–Hydroxy–2–pyrone formation by chalcone and stilbene synthase with nonphysiological substrates. Phytochemistry, **49**: 1945–1951