Plant Molecular Biology **32:** 735–743, 1996.

Chalcone synthase in rice (*Oryza sativa* L.): Detection of the CHS protein in seedlings and molecular mapping of the *chs* locus

Arjula R. Reddy[1], Brian Scheffler[2], G. Madhuri[1], M.N. Srivastava[3], Arvind Kumar[3], P. V. Sathyanarayanan, Suresh Nair and Madan Mohan*

*International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi 110 067, India (*author for correspondence); [1]Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad 500 046, India; [2]Algemeine Botanik, Plant Molecular Biology, University of Hamburg, Hamburg, Germany; [3]Department of Plant Breeding and Genetics, Indira Gandhi Agricultural University, Raipur, India*

Received 12 March 1996; accepted in revised form 29 July 1996

Key words: chalcone synthase cDNA (*chs* cDNA), RFLP, anthocyanins, flavonoids, synteny

Abstract

The chalcone synthase is a key enzyme that catalyses the first dedicated reaction of the flavonoid pathway in higher plants. The *chs* gene and its protein product in rice has been investigated. The presence of a chalcone synthase (CHS) protein in rice seedlings and its developmental stage-specific expression has been demonstrated by western analysis. The chalcone synthase of rice was found to be immunologically similar to that of maize. A rice cDNA clone, *Os-chs* cDNA, encoding chalcone synthase, isolated from a leaf cDNA library of an indica rice variety Purpleputtu has been mapped to the centromeric region of chromosome 11 of rice. It was mapped between RFLP markers RG2 and RG103. RG2 is the nearest RFLP marker located at a genetic distance of 3.3 cM. Some segments of chromosome 11 of rice including *chs* locus are conserved on chromosome 4 of maize. The markers, including *chs* locus on chromosome 11 of rice are located, though not in the same order, on chromosome 4 of maize. Genetic analysis of purple pigmentation in two rice lines, Abhaya and Shyamala, used in the present mapping studies, indicated the involvement of three genes, one of which has been identified as a dominant inhibitor of leaf pigmentation. The *Os-chs* cDNA shows extensive sequence homology, both for DNA and protein (deduced), to that of maize, barley and also to different monocots and dicots.

Introduction

Chalcone synthase catalyses the formation of naringenin chalcone, the first flavonoid carbon-15 skeleton, which is a condensation product of three molecules of malonyl CoA and one molecule of 4-coumaroyl CoA [15]. The CHS is considered a key enzyme as its action is obligatory for the biosynthesis of all flavonoids and anthocyanins in plants. The *chs* gene expression is increased by various biotic and abiotic stress responses in plants [12, 25]. Owing to the vital function of this enzyme, the *chs* gene has been a subject of intensive investigations. The *chs* has been cloned, sequenced and characterized from a number of plant species, including maize [57], parsley [17], *Antirrhinum* [26], *Arabidopsis* [12], *Petunia* [52], bean [45], and *Matthiola* [11].

The molecular analyses of *chs* genes in a wide variety of plant species have revealed that they occur in variable copy number ranging from 1 to 4 in snapdragon, parsley, maize and mustard [5, 36, 41, 47, 57] and from 6 to 10 copies in *Petunia*, pea, French bean and soybean [3, 13, 20, 42, 58]. In addition to such multigenes encoding CHS, several gene fragments and pseudogenes were also identified. For instance, there are at least two gene fragments in addition to 8 copies of complete *chs* genes in *Petunia* strain V30 [20].

Flavonoids in general and CHS in particular have definite functions in plant reproduction [30, 59]. In maize and *Petunia*, presence of functional CHS in anthers was found to be mandatory for male fer-

tility. This condition was first demonstrated in a maize mutant deficient in CHS which produces non-functional white pollen leading to male sterility [9]. In transgenic *Petunia*, a decreased or suppressed *chs* expression by antisense genes was found to result into corollas completely lacking flavonoid pigments [18, 35, 51] and the plants were found to be male-sterile [53]. Interestingly, several other external stimuli like pathogen attack-related elicitor [10], wounding [58] and different light regimes [5] have been shown to induce *chs* transcription in parsley and other plants. Also, CHS mRNA and protein are far more abundant in light-stimulated cells than any other flavonoid pathway genes. The study of *chs* gene in rice assumes importance in view of the overwhelming significance of this gene in flavonoid biosynthesis and its relevance in stress response mechanisms. A growing body of evidence suggests a pivotal role of CHS in more than one biochemical pathway. Elucidation of the role of *chs* expression in different plant functions requires molecular isolation and characterization of the gene and its product. A rice cDNA clone namely, *Os-chs*, hybridizing to the maize c2 gene (encoding chalcone synthase) was isolated from a cDNA library made from developing purple leaves of an indica variety Purpleputtu [38]. Sequence analysis confirmed that the *Os-chs* carries the coding sequence of chalcone synthase [43]. However, the chromosomal position of this gene is not known thus limiting the exploitation of this gene and the pathway in rice research.

We describe here the identification of a CHS protein in seedlings of both indica and japonica rice varieties. The developmental specificity of CHS protein during seedling growth is demonstrated. Further, we mapped the *Os-chs* cDNA on to the rice chromosome using RFLP technique and thus establish the first report of the molecular mapping of a potentially important gene belonging to the anthocyanin pathway in rice. We also demonstrate here the segregation of a putative colour inhibitor in the F_3 mapping population, derived from a cross between two indica rice varieties Abhaya and Shyamala having contrasting phenotypes, green and purple leaves respectively. We report here the *chs* cDNA sequence is conserved among cereals and many other plant species.

Materials and methods

Plant materials

The details of rice lines used in the present study were described earlier [39]. The rice lines were grown either in the field or in pot cultures. For the mapping experiment, 184 F_3 lines were derived from a cross between two indica rice lines Abhaya (green leaves) and Shyamala (purple leaves). This population was primarily raised for mapping of a gene/s that confers resistance to many biotypes of gall midge. Abhaya is the resistant and Shyamala the susceptible parent. The scoring for purple pigmentation of leaves in F-3 lines was carried out when the plants were reaching the heading stage.

RFLP probes

A total of 25 single-copy DNA probes distributed over chromosomes 4, 8 and 11 of rice were randomly selected for the RFLP analysis. These clones (the RG series) were originally selected from a *Pst*I genomic library of rice [27]. In addition, we also used the chalcone synthase cDNA probe which was isolated from a cDNA library derived from an indica rice variety Purpleputtu [38, 43].

Anthocyanin induction, extraction and analysis

Growth of Abhaya and Shyamala seedlings, light induction, anthocyanin pigment extraction and estimations were according to Reddy *et al.* [40]. The extracted pigments were qualitatively analysed by standard chemical methods and TLC using the solvent system described in Reddy *et al.* [40] and Reddy *et al.* [39].

Western analysis

Proteins from leaf blades of young seedlings (30-day-old pot culture) of various rice lines were extracted, estimated (concentration) and electrophoresed in SDS-PAGE according to the standard procedures. The electrophoresed proteins were transferred to membranes and subjected to immunogold blotting using anti-Zm:CHS:C2 antibodies according to the manufacturer's specifications. The anti-Zm:CHS:C2 antibodies were raised against the *c2* gene encoding chalcone synthase in *Zea mays* (a gift from Professor Udo Wienand, AMPI, Hamburg).

The Os-chs *cDNA clone*

The *Os-chs* cDNA clone was originally isolated from a cDNA library constructed from poly(A)$^+$ mRNA of young developing purple leaves of 30-day-old Purpleputtu line in lambda NM1149 and selected by plating the packaged phages onto POP136 cells as in Schwarz-Sommer *et al.* [46]. The *Zm:chs:c2* cDNA was used as the probe [38]. The *Os-chs* cDNA clone was further characterized and sequenced [43]. This *Os-chs* cDNA clone was used in the present mapping experiments.

DNA extraction and Southern hybridization

DNA from the parental lines Abhaya, Shyamala and 184 F_3 progeny lines was isolated by CTAB method of Murray and Thompson [33]. In the initial survey of parental DNAs for RFLPs the following 8 restriction enzymes were used: *Alu*I, *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Hin*dIII, *Not*I and *Xba*I. Another set of 13 restriction enzymes were used to digest parental DNAs if the initial set of eight restriction enzymes failed to reveal RFLPs with a particular probe: *Aat*II, *Ava*I, *Bcl*I, *Cla*I, *Hin*cII, *Kas*I, *Kpn*I, *Msc*I, *Nco*I, *Nde*I, *Nla*IV, *Sal*I and *Xho*I. Genomic DNA restriction, Southern transfer and hybridization were as described earlier [31].

Computing

The MAPMAKER Macintosh Version 1.0 program (E.I. duPont de Nemours and Company, Copyright 1990) based on the MAPMAKER program of Lander *et al.* [24] was used for analysis of segregation data. A BLAST search was done and sequence alignments were on the basis of CLUSTAL method.

Chromosome nomenclature

The numbering system used in this paper is that of McCouch and Tanksley [28].

Results

Detection of a CHS protein in rice seedling extracts

The presence of chalcone synthase was demonstrated by western analysis using anti-Zm:CHS:C2 antibodies. Leaf blade was chosen for this study because of its visible red/purple colour phenotype. Whiteputtu was used as a colourless control. Maize CHS was used as positive control. Our results (Fig. 1A) indicate that the CHS of rice is immunologically similar to that of maize and has an apparent molecular mass of 43–45 kDa, similar to the maize CHS. Of the eight lines tested Purpleputtu leaf extracts show the maximum signal. Signal intensity varied from very faint to almost invisible among other lines. It should be noted that all lines except Whiteputtu showed a red/purple phenotype. Further, the intensity of signal was also found to vary during different developmental stages of Purpleputtu seedlings (Fig. 1B). In young leaves, signal intensity was strong compared to that of old leaves. In some lines, two bands of almost the same molecular mass were observed in certain tissues such as leaf sheath (data not included).

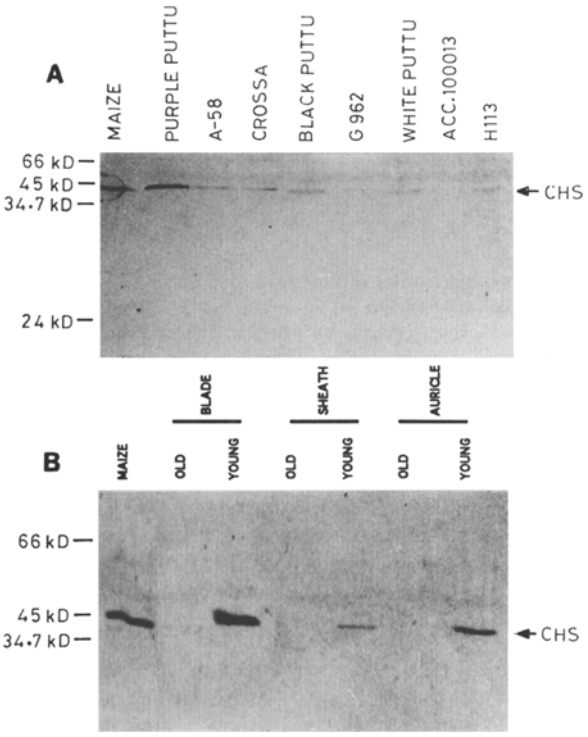

Figure 1. Western blot analysis of expression of CHS protein in young leaves of different rice varieties (arrow). B. Developmental stage-specific expression of CHS proteins in young Purpleputtu seedlings (arrow). Maize CHS was the positive control. Numbers on the left represent the molecular mass markers in kDa.

Genetic and biochemical characterization of parental lines

The data in Table 1 show that the F_1 plants obtained from the reciprocal crosses between parental lines Abhaya (green leaves) and Shyamala (purple leaves)

Table 1. Genetic analysis of purple pigmentation in F_2 plants.

Rice line	Purple	Green	Total	χ^2
Shyamala	+	–	NA	NA
Abhaya	–	+	NA	NA
Shyamala × Abhaya	–	+		
Abhaya × Shyamala	–	+		
F_2 plants	70	363	433	1.58

NA, not applicable; + presence of colour; – absence of colour. The probable genotype of Shyamala is *AACCPPi-Pli-Pl* and that of Abhaya is *AAccppI-PlI-Pl* or *aaCCppIPlIPl* (C, chromogen; A, activator; P, distributor of colour; I-Pl, inhibitor of pigmentation of leaf).

are green indicating the presence of an inhibitory gene in the non-pigmented parent, Abhaya. The F_2-phenotypic ratios (363 purple : 70 green) further suggest that Abhaya is homozygous for a dominant inhibitor of leaf colour. Evidently the Shyamala genotype carries a recessive allele of the putative inhibitor. It is, however, yet to be established if the inhibitor allele present in these lines is allelic to the known I-Pl series. Also it is not clear if Abhaya is a mutant at the *chs* locus or at any other loci of the anthocyanin pathway. The F_2 segregation ratio, 55 (green): 9 (purple), clearly reveals that the parental genotypes differ at three loci, two basic anthocyanin genes and an inhibitor gene. The nomenclature of the genotypes of these lines is based on the CAP system (C, chromogen; A, activator; P, distributor of colour) of genetic control of anthocyanin pigmentation in rice [49]. The tentative genotypes that can be derived are: AA CC PP i-Pli-Pl for Shyamala and AA cc pp I-Pl I-Pl or aa CC pp I-Pl I-Pl for Abhaya (Table 1). In any event, the segregation of a dominant inhibitor is clear. However, genotyping these lines requires further studies.

The spectral, chemical and TLC properties of the anthocyanin pigments accumulated in leaf blades of Shyamala were found to be identical to that of Purpleputtu described in our earlier reports [39, 40]. Shyamala leaves accumulate the major anthocyanidin, cyanidin, and the minor one, peonidin (data not shown). On the other hand, Abhaya leaves do not accumulate detectable levels of anthocyanin pigments and are phenotypically green.

Molecular mapping of the chs *locus*

Molecular mapping was performed on a F_3 population derived from the cross between Abhaya and Shyamala with contrasting phenotypes, green and purple, respectively. RFLP and subsequent co-segregation analysis of the RFLP markers coupled with the co-segregation of *Os-chs* cDNA probe with green/purple pigmented phenotype established the linkage relationship. Using MAPMAKER program [24], we observed that *chs* cDNA co-segregated with RFLP markers from chromosome 11 indicating that the *chs* gene is linked to markers RG2, RG16 and RG103 (Fig. 2A, B) on chromosome 11. *chs* is placed between RFLP markers RG2 and RG103 situated 3.3 cM away from the former and 15.0 cM from the latter (Fig. 2A). While mapping the *chs* locus in rice, we noticed that the *Os-chs* cDNA hybridized to three fragments as revealed by Southern analysis. Thus, the *chs* locus seems to be in two or three copies in rice genome. In the case where the genomic DNAs were restricted with *Eco*RI, the probe hybridized to 3 fragments (data not shown). Two of the fragments (7 kb and 8 kb) seemed to be monomorphic between the two parents. The third band (19 kb in Abhaya, 17 kb in Shyamala) was polymorphic between the parents and it was this fragment that was eventually mapped. This segregating fragment (locus) was mapped on to chromosome 11. It was not possible to map the other two fragments of *chs* as we failed to detect polymorphism between the parents even after screening with 21 different restriction enzymes.

Maize-rice synteny in the chs *region*

When compared with the existing mapping data of *chs* in maize it was observed that some portions of chromosome 11 of rice including *chs* locus are conserved on chromosome 4 of maize (Fig. 2E). The markers including *chs* gene on chromosome 11 of rice are segregating in a similar fashion but not exactly in the same order on chromosome 4 of maize. The region of 12–20 cM from marker RG103-*chs*-RG2-RG16 in rice is conserved in maize (Fig. 2) [2, 4].

Os-chs *cDNA sequence comparison: evolutionary relationship*

The full-length cDNA sequence of *Os-chs* was analyzed using the NCBI GenBank database. About a hundred entries, both from monocots and dicots, showing sequences nearly identical to that of *Os-chs* cDNA were obtained. Of these, only eleven sequences representing cereals and other monocots (group 1) and dicots (group 2) were subjected to BLAST search using the in-frame coding sequence as the query. The data were

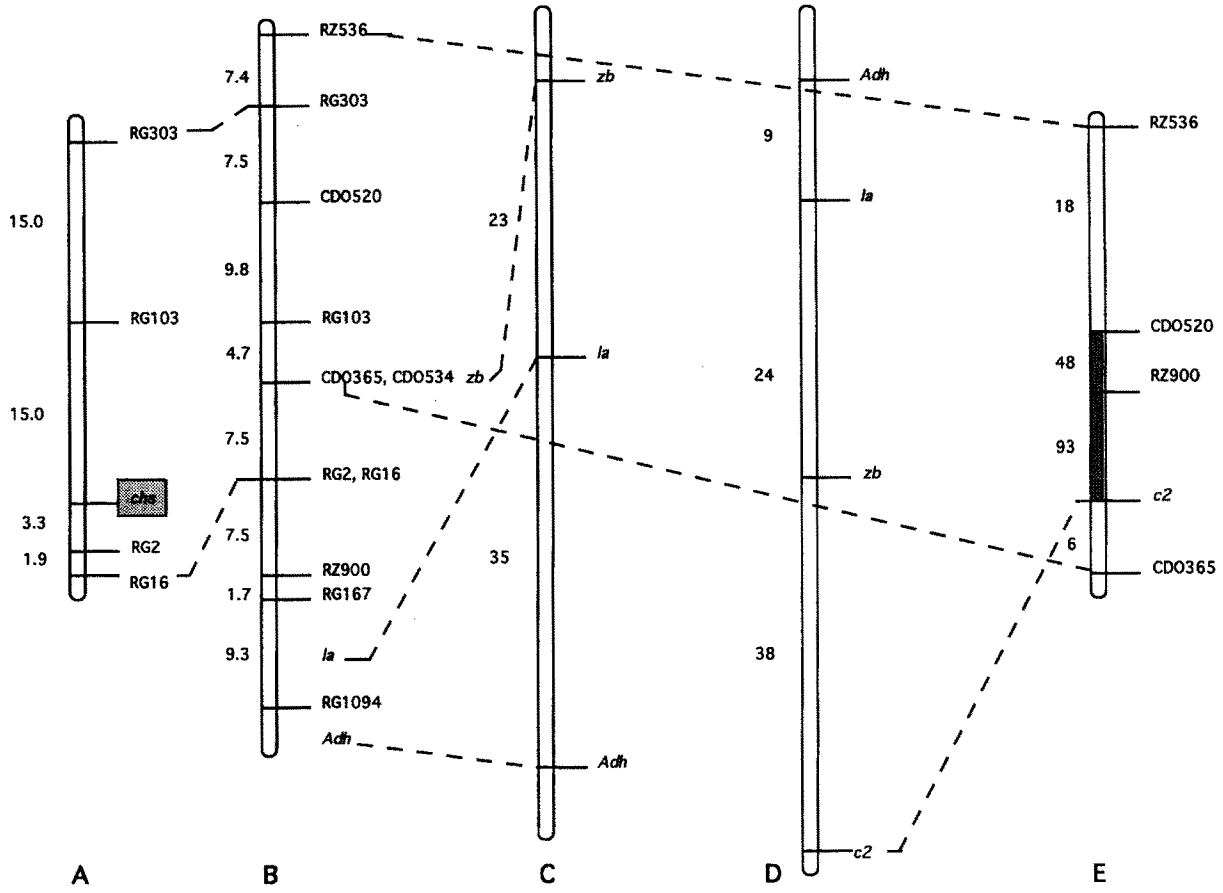

Figure 2. A comparative representation of the linkage map of chromosome 11 of rice and chromosome 4 of maize. A. Partial RFLP map showing the position of *chs* locus (present study). B. A portion of chromosome 11 of rice showing the position of RFLP markers [7]. C. Classical linkage map of chromosome 11 [1]. D. Classical linkage map of chromosome 4 of maize [4]. E. RFLP map of a portion of chromosome 4 of maize showing rice markers [2]. Dotted lines link conserved regions. Shaded portion in the map is not to scale. Locus names: RG, rice random genomic clones; RZ, rice leaf cDNA; CDO, oat leaf cDNA. *zb*, zebra banding; *la*, lazy growth; *Adh*, alcohol dehydrogenase; *c2*, maize chalcone synthase.

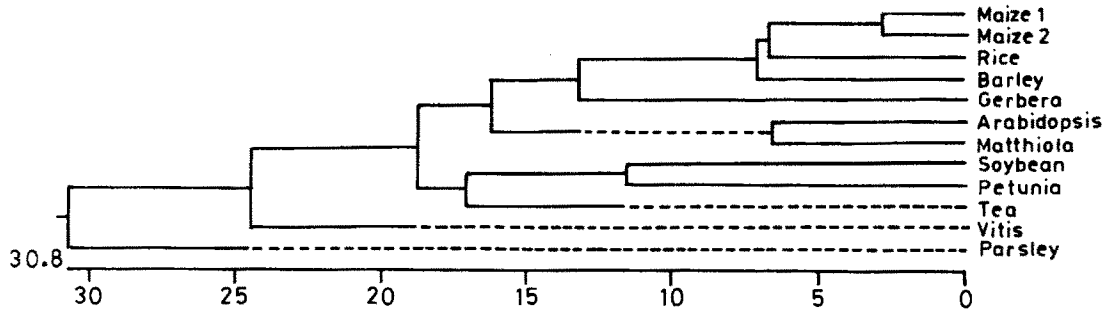

Figure 3. Phylogenetic tree derived using homology between chalcone synthase cDNA sequences of rice to *chs* sequences of different plant species. The accession numbers of the sequences used in homology search are: maize 1, X60204; maize 2, X60205; rice, X89859; barley, X58339; *Gerbera*, Z38096; *Arabidopsis*, M20308; *Matthiola*, X17577; soybean, M98871; *Petunia*, X04080; tea, D26594; *Vitis*, X75969; parsley, V01538. Figures at the bottom show the average distance between clusters.

used to construct a phylogenetic tree (Fig. 3). It is obvious that there is more than 80% identity between *chs* sequences of rice-maize-barley cereal group. It is interesting that barley (*Hordeum vulgare*) shows highest sequence identity (ca. 87%) followed by *Zm:c2: chs* (84.5%) and *Zm:whp:chs* (82.2%) of *Zea mays*. The *c2* and *whp* genes are duplicate genes located on different chromosomes of maize. The nucleotide alterations in *chs* sequences span across the entire coding sequence. The deduced protein sequence comparisons also revealed such an extensive homology between rice, maize and barley chalcone synthases (data not shown). *chs* cDNA sequence comparisons between rice and dicots revealed sequence identity ranging from 60 to 74%.

Discussion

We have identified a CHS protein in seedling extracts of a number of rice lines. The observed differences in CHS protein levels (from western blots) among the tested lines, both indica and japonica types, could not be explained due to the varying genetic background of these lines. Interestingly, Purpleputtu which was originally selected for intense red/purple phenotype of almost all aerial plant parts except node, turned out to be the one showing the maximum expression of CHS protein (Fig. 1A). Two interesting observations can be made from the western data. Firstly, all the tested genotypes except Whiteputtu exhibit red/purple phenotype and yet show enormous differences in CHS levels. Secondly, Whiteputtu having a green (non-purple) phenotype also accumulates detectable levels of CHS protein. Furthermore, we earlier showed that Whiteputtu does not accumulate any anthocyanins [39]. It follows that the *chs* gene is active in Whiteputtu although anthocyanin biosynthesis is blocked in this plant due to a genetic change at any of the other anthocyanin loci. We interpret that the observed differences among rice lines reflect the inherent variation in regulatory mechanisms that govern the co-ordinated expression of genes and gene families of the anthocyanin pathway. The observation that young leaves have more CHS protein than the old leaves of Purpleputtu reflects developmental specificity of CHS expression and regulation (Fig. 1B). Instances where the individual members of *chs* multigene family are selectively expressed at different developmental stages in particular tissues or organs include maize, *Petunia*, parsley and bean [8, 21, 29, 44, 52]. Tissue-specific expression of *chs* has also been reported in flowers where a flower-specific *chs* promoter was first identified in *Antirrhinum* [47] and later in *Petunia hybrida* [22]. *chs* promoter analysis in transgenic *Petunia* plants revealed DNA sequences that direct anther specific expression [55]. Promoter elements of *chs* genes conferring specificity of expression in different organs have also been described [57, 59]. Recently, it has been shown that *chs*-like genes in plants have evolved into a supergene family whose members have both different regulation and capacity to code for different but related enzymatic activities [14]. It would be of further interest to detect the spatial regulation of the gene and the regulatory factors governing it.

We have mapped one copy of *chs* gene on to chromosome 11 of rice between the RFLP markers RG2 and RG103 (Fig. 2A). RG2 is closely linked to *chs* gene at a distance of 3.3 cM. The RG2 locus is an important locus and has been previously shown to be highly heterozygous in recombinant inbred lines of rice and could be potentially contributing to heterosis [34]. RG2 has also been physically mapped in the centromeric region of chromosome 11 of rice [48] where mapping is hampered by the relative low levels of polymorphism. Presently, we are unable to map the other copy/ies of the *chs* gene in rice. Further work will be required to determine whether the other copy/ies of *chs* gene are located close to the present mapped one or at a different location on the same chromosome or on to a different chromosome. In maize, different copies of *chs* gene are found on different chromosomes [8].

Comparative maps based on RFLPs have revealed that the genomes of tomato and potato are nearly identical in overall gene content and gene order [6, 50]. Similarly, conserved linkages have been reported between maize and sorghum [16, 56]; and wheat and rice [23]. If there are similarities between two species in linkage conservation, then genetic information and molecular probes from one species can be utilized in another. The results would contribute to a faster mapping research on all species being compared. It is now possible to find out which regions of the genome are homoeologous among cereals by using molecular probes from one crop to screen genome of another crop. Comparative linkage maps of rice and maize genomes support the view that centromere position is often conserved [2]. Van Deynze *et al.* [54] have found that the orders of markers detected by probes in rice, maize and oat are conserved. Kilian *et al.* [19] have found a high degree of synteny between the telomeric regions of barley chromosome 1P and rice chromosome 6. It is

interesting to know that in the present study, the markers on chromosome 11 of rice are located in a similar fashion but not in the same order, on chromosome 4 of maize. One of the chalcone synthase genes, the *c2* in maize is mapped on to chromosome 4 [8]. The molecular marker pairs RZ536/CD0520 (rice chromosome 11; maize chromosome 4) are also linked (<20cM) and are in single copy in both genomes [2]. It is also pertinent to mention that a portion of chromosome 4 and 7 in maize is duplicated and thus both these maize chromosomes could have synteny with chromosome 11 of rice [32].

We have identified the accumulated anthocyanin pigments and predicted the likely genetic constitution. Shyamala seedlings accumulate cyanidin as the major pigment and peonidin as the minor pigment which is in agreement with our earlier report on the nature of pigments in indica rice lines [37, 39, 40]. The accumulation of anthocyanin pigments in Shyamala is indicative of the presence of the fully functional gene complement of the pathway. On the contrary, the absence of the pigment in Abhaya would reflect the mutant version of the gene or genes in addition to a dominant inhibitor allele. It is not possible to identify the specific mutant alleles at individual loci in the Abhaya genotype. In any event, that the Abhaya genotype is homozygous for an inhibitor of leaf pigmentation has been clearly demonstrated (Table 1). However, it is to be established whether this inhibitor is allelic to the already reported I-Pl or Ilb inhibitors [39].

The *chs* coding sequence is highly conserved across both monocots and dicots. The extensive DNA and protein (deduced) sequence homology of *Os-chs* cDNA with that of maize and barley assumes importance with regard to the conservation of this gene during evolution of cereal genomes and therefore, its possible role in a multitude of biological functions. The phylogenetic relationships can be summarized as follows: maize, rice and barley form into a closely related group with maximum sequence conservation showing more than 80% homology. Dicots form the second group with the sequence identity ranging from 60 to 70% with that of rice (Fig. 3). Though dicots diverged from monocots millions of years ago, the monocot *chs* sequence is highly conserved amongst them. This is in agreement with the earlier report that the CHS protein is highly conserved and the amino acid sequences display 80–90% identity among angiosperms [36].

Acknowledgements

We thank Professor K. K. Tewari, Director, ICGEB, and Professor Udo Wienand, University of Hamburg, for their generous help and constant encouragement. Financial support from the Rockefeller Foundation to University of Hyderabad and International Centre for Genetic Engineering and Biotechnology (ICGEB) is gratefully acknowledged. Thanks are also due to the Directorate of Rice Research, Hyderabad for supply of plant materials. G.M. thanks the Department of Atomic Energy for providing a predoctoral research fellowship. We thank Dr Claude Fauquet, The Scripps Research Institute, California, for help with the phylogenetic analysis.

References

1. Abenes MLP, Tabien RE, McCouch SR, Ikeda R, Ronald P, Khush GS Huang N: Orientation and integration of the classical and molecular genetic maps of chromosome 11 in rice. Euphytica 76: 81–87 (1994).
2. Ahn S, Tanksley SD: Comparative linkage maps of the rice and maize genomes. Proc Natl Acad Sci USA 90: 7980–7984 (1993).
3. Akada S, Dube SK: Organization of soybean chalcone synthase gene clusters and characterization of a new member of the family. Plant Mol Biol 29: 189–199 (1995).
4. Anonymous: In: Maize Genet Coop Newsl p. 250 (1995).
5. Batschauer A, Ehmann B, Schafer E: Cloning and characterization of a chalcone synthase gene from mustard and its light dependent expression. Plant Mol Biol 16: 175–185 (1991).
6. Bonierbale M, Plaisted RL, Tanksley SD: RFLP maps based on a common set of clones reveal modes of chromosomal evolution in potato and tomato. Genetics 120: 1095–1103 (1988).
7. Causse MA, Fulton TM, Cho YG, Ahn SN, Chunwongse J, Wu K, Xiao J, Yu Z, Ronald PC, Harrington SE, Second G, McCouch SR, Tanksley SD: Saturated molecular map of the rice genome based on an interspecific backcross population. Genetics 38: 1251–1274 (1994).
8. Coe EH, Neuffer MG, Hoisington DA: The genetics of corn. In: Sprague GF, Dudley JW (eds) Corn and Corn Improvement, pp. 81–258. American Society of Agronomy, Madison, WI (1988).
9. Coe EH, McCormick S, Modena SA: White pollen in maize. J Hered 72: 318–320 (1981).
10. Dangl JL, Hahlbrock K, Schell J: Regulation and structure of chalcone synthase genes. In: Vasil IK, Schell J (eds) Cell Culture and Somatic Cell Genetics of Plants, vol. 4, pp. 155–173. Academic Press, New York (1989).
11. Epping B, Kittel M, Ruhnau B, Hemleben V: Isolation and sequence analysis of a chalcone synthase cDNA of *Matthiola incana* R.Br (Brassicaceae). Plant Mol Biol 14: 1061–1063 (1990).
12. Feinbaum RL, Ausubel FM: Trancriptional regulation of the *Arabidopsis thaliana* chalcone synthase gene. Mol Cell Biol 8: 1985–1992 (1988).

13. Harker CL, Ellis THN, Coen ES: Identification and genetic regulation of chalcone synthase multigene family in pea. Plant Cell 2: 185–194 (1990).
14. Helariutta Y, Elomaa P, Kotilainen M, Griesbach RJ, Schroder J, Teeri TH: Chalcone synthase-like genes active during corolla development are differentially expressed and encode enzymes with different catalytic properties in *Gerbera hybrida* (Asteraceae). Plant Mol Biol 28: 47–60 (1995).
15. Heller W, Hahlbrock K: Highly purified flavanone synthase from parsley catalyses the formation of naringenin chalcone. Arch Biochem Biophys 200: 617–619 (1980).
16. Hulbert SH, Richter TE, Axtell JD, Bennetzen JL: Genetic mapping and characterization of sorghum and related crops by means of maize DNA probes. Proc Natl Acad Sci USA 87: 4251–4255 (1990).
17. Jahnen W, Hahlbrock K: Differential regulation and tissue specific distribution of enzymes of phenylpropoanoid pathways in developing parsley seedlings. Planta 173: 197–204 (1988).
18. Jorgensen R: Altered gene expression in plants due to trans interactions between homologous genes. Trends Biotechnol 8: 340–344 (1990).
19. Kilian A, Kudrna DA, Kleinhofs A, Yano M, Kurata N, Steffenson B, Sasaki T: Rice-barley synteny and its application to saturation mapping of the barley Rpg1 region. Nucl Acids Res 23: 2729–2733 (1995).
20. Koes RE, Spelt CE, Mol JNM, Gerats AGM: The chalcone synthase multigene family of *Petunia hybrida* (V30): sequence homology, chromosomal localization and evolutionary aspects. Plant Mol Biol 10: 159–169 (1987).
21. Koes RE, Spelt CE, van den Elzen PJM, Mol JNM: Cloning and molecular characterization of the chalcone synthase multigene family of *Petunia hybrida*. Gene 81: 245–257 (1989).
22. Koes RE, van Blokland R, Quattrocchio F, van Tunen AJ, Mol JNM: Chalcone synthase promoters in *Petunia* are active in pigmented and unpigmented cell types. Plant Cell 2: 379–392 (1990).
23. Kurata N, Moore G, Nagamura Y, Foote T, Yano M, Minobe Y, Gale M: Conservation of genome structure between rice and wheat. Bio/technology 12: 276–278 (1994).
24. Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L: MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1: 174–181 (1987).
25. Lawton MA, Lamb CJ: Transcriptional activation of plant defense genes by fungal elicitor, wounding and infection. Mol Cell Biol 7: 335–341 (1987).
26. Lipphardt S, Brettschneider R, Kreuzaler F, Schell J, Dangl JL: UV-inducible transient expression in parsley protoplasts identifies regulatory *cis*-elements of a chimeric *Antirrhinum majus* chalcone synthase gene. EMBO J 7: 4027–4033 (1988).
27. McCouch SR, Kochert G, Yu ZH, Wang ZY, Khush GS, Coffman WR, Tanksley SD: Molecular mapping of rice chromosomes. Theor Appl Genet 76: 815–829 (1988).
28. McCouch SR, Tanksley SD: Development and use of restriction fragment length polymorphism in rice breeding and genetics. In: Khush GS, Toenniessen GH (eds) Rice Biotechnology, pp. 109–133. CAB International, Wallingford, UK (1991).
29. Meier BM, Shaw N, Slusarenko AJ: Spatial and temporal accumulation of defense genes transcripts in bean (*Phaseolus vulgaris*) leaves in relation to bacteria-induced hypersensitive cell death. Mol Plant-Microb Interact 6: 453–466 (1993).
30. Mo Y, Nagel C, Taylor LP: Biochemical complementation of chalcone synthase mutants defines a role for flavonols in functional pollen. Proc Natl Acad Sci USA 89: 7213–7217 (1992).
31. Mohan M, Nair S, Bentur JS, Prasada Rao U, Bennett J: RFLP and RAPD mapping of the rice *Gm2* gene that confers resistance to biotype 1 of gall midge (*Orseolia oryzae*). Theor Appl Genet 87: 782–788 (1994).
32. Moore G, Devos KM, Wang Z, Gale MD: Grasses, line up and form a circle. Curr Biol 5: 737–739 (1995).
33. Murray MG, Thompson WF: Rapid isolation of high molecular weight plant DNA. Nucl Acids Res 8: 4321–4325 (1980).
34. Nair S, Prasada Rao U, Bennett J, Mohan M: Detection of a highly heterozygous locus in recombinant inbred lines of rice and its possible involvement in heterosis. Theor Appl Genet 91: 978–986 (1995).
35. Napoli C, Lemieux C, Jorgensen R: Introduction of a chimeric chalcone synthase gene into *Petunia* results in reversible cosuppression of homologous genes *in trans*. Plant Cell 2: 279–289 (1990).
36. Niesbach-Klosgen U, Barzen E, Bernhardt J, Rohde W, Schwarz-Sommer Z, Reif HJ, Weinand U, Saedler H: Chalcone synthase genes in plants: a tool to study evolutionary relationships. J Mol Evol 26: 213–225 (1987).
37. Reddy AR: Genetics and molecular analysis of the anthocyanin pigmentation pathway in rice. In: Proceedings of the International Rice Genetics Meetings, Manila, Philippines, 15–21 October (1995).
38. Reddy AR, Saedler AG, Scheffler B, Wienand U: Studies on transposable elements and anthocyanin pathway in rice: gene cloning and characterization. In: Proceedings of the International Program on Rice Biotechnology, Bali, Indonesia, 16–20 May, p. 155 (1994).
39. Reddy VS, Dash S, Reddy AR: Andiocyanin pathway in rice (*Oryza sativa* L.): Identification of a mutant showing dominant inhibition of anthocyanins in leaf and accumulation of proanthocyanidins in pericarp. Theor Appl Genet 91: 301–312 (1995).
40. Reddy VS, Goud K, Sharma R, Reddy AR: Ultraviolet-B-responsive anthocyanin production in rice cultivar is associated with a specific phase of phenylalanine ammonia lyase biosynthesis. Plant Physiol 105: 1059–1066 (1994).
41. Reimold U, Kroeger M, Kreuzaler F, Hahlbrock K: Coding and 3′ non-coding nucleotide sequence of chalcone synthase mRNA and assignment of amino acid sequence of the enzyme. EMBO J 2: 1801–1805 (1983).
42. Ryder TB, Hedrick SA, Bell JN, Liang X, Clouse SD, Lamb CJ: Organization and differential activation of a gene family encoding the plant defense enzyme chalcone synthase in *Phaseolus vulgaris*. Mol Gen Genet 210: 219–233 (1987).
43. Scheffler BE, Reddy AR, Hoffman I, Wienand U: Chalcone synthase cDNA from *Oryza sativa*. Plant Physiol 109: 722 (1995).
44. Schmelzer E, Jahnen W, Hahlbrock K: In situ localization of light-induced chalcone synthase mRNA, chalcone synthase, and flavonoid end products in epidermal cells of parsley leaves. Proc Natl Acad Sci USA 85: 2989–2993 (1988).
45. Schmid J, Doerner PW, Clouse SD, Dixon RA, Lamb CJ: Developmental and environmental regulation of a bean chalcone synthase promoter in transgenic tobacco. Plant Cell 2: 619–631 (1990).
46. Schwarz-Sommer Z, Gierl A, Cuypers H, Peterson PA, Saedler H: Plant transposable elements generate the DNA sequence diversity needed in evolution. EMBO J 4: 591–597(1985).

47. Sommer H, Saedler H: Structure of the chalcone synthase gene of *Antirrhinum majus*. Mol Gen Genet 202: 429–434 (1986).
48. Song YC, Gustafson JP: The physical location of fourteen RFLP markers in rice (*Oryza sativa* L.). Theor Appl Genet 90: 113–119 (1995).
49. Takahashi M: Genetical studies on rice. LXXX. Gene analysis and its related problems. J Fac Agr Hokkaido Univ 61: 91–142 (1982).
50. Tanksley SD, Ganal MW, Prince JP, de Vicente MC, Bonierbale MW, Broun P, Fulton TM, Giovannoni JJ, Grandillo S, Martin GB, Messeguer R, Miller JC, Miller L, Paterson AH, Pineda O, Roder MS, Wing RA, Wu W, Young ND: High density molecular linkage maps of the tomato and potato genomes. Genetics 132: 1141–1160 (1992).
51. Taylor LP, Jorgensen R: Conditional male fertility in chalcone synthase deficient *Petunia*. J Hered 83: 11–17 (1992).
52. van der Meer IM, Spelt CE, Mol JNM, Stuitze AR: Promoter analysis of chalcone synthase (*chsA*) gene of *Petunia hybrida*: a 67 bp promoter region directs flower-specific expression. Plant Mol Biol 15: 95–109 (1990).
53. van der Meer IM, Stam ME, van Tunen AJ, Mol JNM, Stuitze AR: Antisense inhibition of flavonoid biosynthesis in *Petunia* anthers results in male sterility. Plant Cell 4: 253–262 (1992).
54. van Deynze AE, Nelson JC, Yglesias ES, Harrington SE, Braga DP, McCouch SR, Sorrells ME: Comparative mapping in grasses. Wheat relationships. Mol Gen Genet 248: 744–754 (1995).
55. van Tunen AJ, Koes RE, Spelt CE, van der Krol AR, Stuitje AR, Mol JNM: Cloning of the two chalcone flavanone isomerase genes from *Petunia hybrida*: coordinate, light regulated and differential expression of flavonoid genes. EMBO J 7: 1257–1263 (1988).
56. Whitkus R, Doebley J, Lee M: Comparative genome mapping of sorghum and maize. Genetics 132: 1119–1130 (1992).
57. Wienand U, Weydemann U, Niesback-Klosgen U, Peterson PA, Saedler H: Molecular cloning of the *c2* locus of *Zea mays*, the gene coding for chalcone synthase. Mol Gen Genet 203: 202–207 (1986).
58. Wingender R, Rohrig H, Horicke C, Wing D, Schell J: Differential regulation of soybean chalcone synthase genes in plant defense, symbiosis and upon environmental stimuli. Mol Gen Genet 218: 315–322 (1989).
59. Ylstra B, Busscher J, Franken J, Hollman PCH, Mol JNM, Tunen AJV: Flavonoids and fertilization in *Petunia hybrida*: localization and mode of action during pollen growth. Plant J 6: 201–212 (1994).