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Isolation of gibberellin metabolic pathway genes from barley and comparative mapping in barley, wheat and rice

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Abstract Gene sequences encoding gibberellin (GA) biosynthetic and catabolic enzymes were isolated from 'Himalaya' barley. These genes account for most of the enzymes required for the core pathway of GA biosynthesis as well as for the first major catabolic enzyme. By means of DNA gel blot analysis, we mapped coding sequences to chromosome arms in barley and wheat using barley-wheat chromosome addition lines, nulli-tetrasomic substitution and ditelosomic lines of wheat. These same sequences were used to identify closely related sequences from rice, which were mapped in silico, thereby allowing their syntenic relationship with map locations in barley and wheat to be investigated. Determination of the chromosome arm locations for GA metabolic genes provides a framework for future studies investigating possible identity between GA metabolic genes and dwarfing genes in barley and wheat.

Introduction

Gibberellins (GA) comprise a large hormone family that modifies many aspects of plant growth and development. In plants, GA biosynthesis is catalysed by three enzyme classes specific to different steps: (1) the terpene cyclases, *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS), catalyse the initial cyclisation of geranylgeranyldiphosphate to *ent*-kaurene; (2) intermediate steps in the pathway are catalysed by cytochrome P450 monooxygenases, including *ent*-kaurene oxidase (KO) and *ent*-kaurenoic acid oxidase (KAO); (3) the late steps that result in the synthesis of active GAs are catalysed by 2-oxoglutarate-dependent dioxygenases, including 20-oxidase (20ox) and 3-oxidase (3ox). The first catabolic step is also catalysed by a dioxygenase, the 2-oxidase (2ox). Genes for each of these steps have been isolated across a range of plant species and their identity confirmed by approaches that include the characterisation of mutants, transgenic plants and the in vitro analysis of protein activity (reviewed by Hedden and Kamiya 1997). DNA sequences related to these genes are present in many public databases, although it is not always clear whether these encode enzymes of GA metabolism or related enzymes that catalyse non-GA reactions.

Yield improvements of major cereal crop species have been accompanied by a reduction in plant height. In rice, reduced height was achieved by introducing a major dwarfing gene (*sd-1*) that encodes a defective GA 20ox gene, thereby affecting the late steps in the GA biosynthetic pathway (Ashikari et al. 2002; Monna et al. 2002; Spielmeyer et al. 2002). In the investigation reported here, we isolated GA biosynthetic and catabolic genes from barley and located these genes to chromosome arms in barley and wheat as a first step in determining whether any dwarfing genes in barley and wheat correspond to defective GA metabolic genes. Barley genes were used to identify closely related sequences from rice with the view to assemble all potential rice GA metabolic genes. This information may assist us in the future to isolate additional GA metabolic genes from barley and wheat. We mapped in silico rice GA metabolic genes and closely related sequences and investigated possible orthologous relationships to the corresponding barley genes based on sequence comparison and syntenic map locations.

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Materials and methods

Barley sequences related to GA biosynthetic and catabolic genes

The clones that form the basis for this study were isolated from two different cDNA libraries prepared from Himalaya barley (*Hordeum vulgare* L.). One library was derived from RNA of developing endosperm (10–15 days post-anthesis; Ali et al. 2000), and this was probed with DNA from a variety of sources. The following full-length coding sequences (and the respective probe) were isolated from the endosperm cDNA library: HvCPSL1 (maize CPS), HvKSL1 (barley EST AV836032), Hv20ox3 (wheat 20ox), and Hv3ox1 (clone identified by random sequencing of inserts). Another clone from this library, HvKO1 (isolated using a KO probe from arabidopsis) is near full-length, lacking an estimated 30 amino acid residues from the NH_2 terminus of the protein. The second cDNA library was prepared from elongating leaves of seedlings. This was screened with DNA probes from several sources, including clones from the endosperm library. The following full-length coding sequences were isolated from the leaf cDNA library: Hv20ox1 (wheat 20ox, barley Hv20ox3), Hv3ox2 (sequences from wheat 3ox) and Hv2ox4 and Hv2ox5 (sequences from barley EST AL505365). In addition, a partial sequence, HvKSL2, which includes approximately 540 nucleotides from the 3′ end of a KS-related gene was amplified from Himalaya barley DNA using primers designed from barley EST AV836032.

Functional assay for dioxygenases

Dioxygenase clones were isolated in either pSport (GibcoBRL, Gaithersburg, Md.) or Bluescript (Stratagene, La Jolla, Calif.) vectors and were expressed using the TNT Coupled Reticulocyte Lysate kit (Promega, Madison, Wis.). One microgram of plasmid DNA, linearised at the 3′ end of the insert, was incubated at 30°C with either T3 or T7 RNA polymerase (as appropriate) to generate RNA that was translated in vitro. After a 90-min incubation, 20 µl of 10× cofactor mix (40 m*M* 2-oxoglutarate, 40 m*M* ascorbate, 5 m*M* $FeSO_4$) and 20 µl of catalase solution (10 mg ml^{-1}) were added to each reaction, along with 20 Kdpm of the appropriate [17-^{14}C] GA substrate. Incubation continued at room temperature for a further 60 min, and the reaction was stopped by the addition of 5 µl glacial acetic acid and 1 ml acetone. The sample was centrifuged at 20,000 *g* for 5 min, the supernatant dried and GAs analysed by reverse-phase [C_{18}] high-performance liquid chromatography (HPLC) as previously described (Green et al. 1997). Radioactivity in half of each fraction was determined by liquid scintillation counting, and selected remaining halves were pooled according to the positions of the radioactive peaks. The samples were dried, derivatised and analysed by gas chromatography (GC)-selected ion monitoring (SIM) as described (Green et al. 1997). GA products were identified on the basis of their retention time on HPLC and GC and by characteristic spectra of M/M+2 and backup ions.

DNA probes derived from GA biosynthetic and catabolic genes

DNA probes used in this study contained either the whole coding sequence or, in some cases, were selected from 3′ regions to provide additional hybridisation specificity. Probes from the 3′ ends of HvKSL1 and HvKSL2 shared 70% nucleotide sequence identity and weakly cross-hybridised under our conditions. The HvKO1 and HvKAO1 sequences were mapped using DNA probes derived from the 3′ end of the respective sequences. The 3′ probes derived from Hv20ox1 and Hv20ox3 sequences were only 50% identical and did not cross-hybridise. Probes derived from the 3′ end of Hv3ox1 and Hv3ox2 sequences were 78% identical and showed some cross-hybridisation. The 3′ probes for related gene members Hv2ox4 and Hv2ox5 showed weak cross-hybridisation.

DNA hybridisation and chromosomal localisation

Chromosomal locations of restriction fragment length polymorphism (RFLP) markers in barley were determined by probing genomic DNA (approximately 12 µg) of wheat-barley chromosome addition lines (Islam et al. 1981) that had been cut with *Dra*I, *Eco*RI, *Eco*RV and *Hin*dIII restriction enzymes, gel-fractionated and blotted and immobilised on nylon membranes. Some genes were located on chromosome arms in barley by probing ditelosomic wheat-barley addition lines (Islam et al. 1981). In wheat, nullitetra-disomic and ditelosomic aneuploid stocks developed in Chinese Spring (Sears 1954) were used to determine the chromosome arm locations of RFLP markers detected by the probes. Hybridisation was carried out in aqueous buffer at 65°C. The membranes were washed at 65°C twice, 20 min each time, in 2× SSC/0.1% sodium dodecyl sulphate (SDS), once for 20 min in 1× SSC/0.1% SDS and once for 15 min in 0.5× SSC/0.1% SDS.

Database searches and sequence alignment

Several public databases were used for the comparison between barley and rice:

1. Closely related rice protein sequences were identified by using a protein query against the translated rice database (TBLASTN, non-redundant and high-throughput genomic sequences) hosted by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) or the Gramene database (http://www.gramene.org/index.html). The statistical significance threshold for reporting matches against database sequences was set at E-value −40 but was increased to E-value −80 for searches using monooxygenase sequences.
2. Rice PAC/BAC (bacterial/P1-derived artificial chromosome) clones containing predicted genes were positioned on the physical map and tightly linked markers identified using the FINGERPRINTED CONTIGS (FPC) in the RICE GENOME BROWSER on Gramene (http://www.gramene.org/index.html).
3. Syntenic regions were determined between wheat and rice on the basis of common cDNA markers using the COMPARATIVEMAP VIEWER on Gramene (http://www.gramene.org/cmap/viewer).
4. The predicted gene sequences were identified within BAC/PAC clones by using the Rice Genome Automated Annotation System (http://ricegaas.dna.affrc.go.jp) or the GENOME VIEWER on Gramene (http://www.gramene.org/perl/contigview).
5. The phylograms were generated using CLUSTALW with default settings at the site of the European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw/). The multiple sequence alignments were made using amino acid sequences for enzymes involved in GA metabolism in other species but also included related enzymes that catalyse non-GA reactions.

Results

Isolation of barley genes

The major steps in the biosynthesis and initial catabolism of GA are summarised in Fig. 1. cDNA clones related to each of the enzymes were isolated from barley (Table 1) and have been characterised by sequence comparisons, analysis of dwarf mutants and in vitro enzyme activity. In four instances (HvKSL, Hv20ox, Hv3ox, Hv2ox) there were two closely related sequences isolated, presumably encoding isozymes. There is a moderate level of amino acid sequence relatedness of the barley sequences to the representative enzymes for that step (see Table 1). HvCPSL1 (CPS-like) and the two HvKSL (KS-like)

sequences have been characterised only on the basis of sequence. They cluster preferentially with the terpene cyclases involved in GA metabolism rather than with other terpene cyclases that are not involved in GA metabolism (Fig. 2a). Similarly, HvKO1 and HvKAO1 cluster preferentially with the cytochrome P450 monooxygenases involved in GA biosynthesis rather than with related enzymes not involved in GA metabolism (Fig. 2b). There is additional evidence for the roles of HvKO1 and HvKAO1 in GA biosynthesis in barley. Mutants at the *grd3* dwarfing locus in barley are deficient in endogenous GA; they accumulate large quantities of the intermediate *ent*-kaurene, and have mutations in the HvKO1 sequence (P.M. Chandler, J.L. Radik, J.R. Lenton, unpublished). The HvKAO1 gene is the *Grd5* dwarfing locus in barley and produces a functional enzyme in vitro (Helliwell et al. 2001). The six dioxygenase sequences (Hv20ox1, Hv20ox3, Hv3ox1, Hv3ox2, Hv2ox4 and Hv2ox5) cluster with other dioxygenases involved in GA metabolism, and their nomenclature (ox1, -2, -3 etc.) is aimed at uniformity for orthologous genes from barley, wheat and rice. The enzymes encoded by the clones were assessed for activity with in vitro-synthesised protein and radio-labelled GA substrates (Table 2). In all cases the products from the dioxygenase genes catalysed the expected reactions in vitro. There were differences between clones in the efficiency of conversion and between isozyme pairs in their substrate preferences; especially notable was the extreme difference in specificity shown by the two Hv2ox isozymes.

Chromosome mapping

DNA probes allowed us to localise each barley gene to a specific chromosome or chromosome arm in barley and wheat. It was often necessary to use probes from the 3′ end to enhance specificity, especially for closely related sequences. The mapping results for each gene are given in detail below and are summarised in Table 3. A representative set of blots is shown for one gene (*HvKSL2*) and described in detail in the legend of Fig. 3.

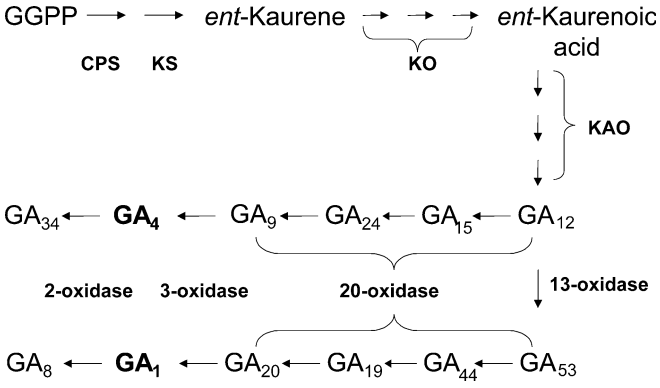

Fig. 1 Simplified GA biosynthetic pathways occurring in higher plants. GA_1 and GA_4 are biologically active GAs which are catabolised to inactive GA_8 and GA_{34}, respectively. The 13-oxidase gene responsible for the initial conversion of GA_{12} to GA_{53} has not yet been cloned. *GGPP* Geranylgeranyldiphosphate, *CPSent*-copalyl diphosphate synthase, *KSent*-kaurene synthase, *KO ent*-kaurene oxidase, *KAO ent*-kaurenoic acid oxidase

ent-Copalyl diphosphate synthase

The HvCPSL1 probe gave at least two strongly hybridising bands derived from chromosome 7H of barley and homoeologous bands derived from the long arm of chromosome group 7 of wheat. The same probe also detected several weakly hybridising bands in barley and wheat that mapped to the short arm of chromosome group 2. A Genbank search using *HvCPSL1* and a previously isolated CPS gene from maize (L37750) identified one closely related rice sequence on the short arm of chromosome 2 (R1) and another on the long arm of chromosome 9 (R2) of rice (Fig. 2a). The alignment of protein sequences showed that HvCPSL1 is more closely related to the R1 sequence (72% identity) than to R2 (59% identity) (Fig. 2a). Using the Gramene database we identified a small syntenic region on chromosome 7HL of barley and the short arm of chromosome 2 of rice suggesting that *HvCPSL1* in barley is an orthologue of *R1* in rice (Fig. 4a).

ent-Kaurene synthase

The HvKSL1 probe hybridised to multiple fragments, with the strongest signal mapping to the long arm of chromosome 2H in barley and chromosome group 2 in wheat (Table 3). The HvKSL2 probe was more specific and gave one strong band and several weak bands on chromosome 2HL in barley; the same probe detected three homoeologous genes on the long arm of chromosome group 2 in wheat (Fig. 3). The HvKSL1 and HvKSL2 probes shared 70% overall sequence identity, and weak cross-hybridisation was observed, but polymorphic bands could be assigned either to the *HvKSL1* or *HvKSL2* gene on the basis of relative hybridisation intensity. The HvKSL1 protein sequence detected four closely related rice sequences in the Genbank database (Fig. 2a), one of which (OsKS1A) had been previously identified (Genbank Accession no. AY347876). Based on CLUSTALW analysis of amino acid sequences it is likely that the sequences OsKS1A, R3, R4 and R5 encode KS genes. Two genes (*OsKS1A* and *R4*) were present within 25 kb of the DNA sequence on the long arm of chromosome 4, another sequence (*R3*) was located on the short arm of chromosome 4, whereas the fourth gene member (*R5*) was positioned on the long arm of chromosome 2 (Fig. 4a). Synteny between chromosome 2HL of barley and the long arm of chromosome 4 of rice suggests that genes within these regions may be orthologous (Fig. 4a). Although all of the polymorphic HvKSL bands in wheat and barley were located on chromosome 2L, we are unable to say whether they are closely linked as in rice.

Table 1 Barley cDNA sequences, accession numbers and their deduced amino acid sequence relationships to representative enzymes of GA biosynthesis and catabolism

Enzyme	Representative locus, accession number, reference	Barley cDNA sequence[a]	Accession number	Amino acid sequence identity to representative (%)
CPS (copalyl phosphate synthase)	Maize *An1*, T02959, Bensen et al. (1995)	HvCPSL1	AY551435	65
KS (*ent*-kaurene synthase)	Arabidopsis *GA2*, AF034774, Yamaguchi et al. (1998)	HvKSL1	AY551436	46
		HvKSL2	AY551437	
KO (*ent*-kaurene oxidase)	Arabidopsis *GA3*, AF047719, Helliwell et al. (1998)	HvKO1	AY551434	60
KAO (*ent*-kaurenoic acid oxidase)	Barley *Grd5*, AF326277, Helliwell et al. (2001)	HvKAO1	AF326277	100
20-Oxidase	Arabidopsis *GA5*, U20872, Xu et al. (1995)	Hv20ox1	AY551428	63
		Hv20ox3	AY551429	54
3-Oxidase	Rice, AB056518, Itoh et al. (2001)	Hv3ox1	AY551430	59
		Hv3ox2	AY551431	73
2-Oxidase	*Phaseolus coccineus*, AJ132438, Thomas et al. (1999)	Hv2ox4	AY551432	53
		Hv2ox5	AY551433	47

[a]Clones include the full coding regions with the exceptions of HvKO1, which lacks an estimated 30 amino acids at the NH_2 terminus and HvKSL2, which represents only the COOH-terminal 180 amino acids

Table 2 Metabolism of radio-labelled GAs by in vitro transcription/translation products of dioxygenase clones (*ND* none detected)

Clone	[^{14}C] substrate	[^{14}C] product(s)[a] (% input radioactivity)
Hv20ox1	GA_{53}	GA_{44} (10)
	GA_{19}	GA_{20} (50); GA_{17}[2] (trace)
Hv20ox3	GA_{53}	$GA_{44,\ 19}$ (80); GA_{20} (20)
	GA_{19}	GA_{20} (80); GA_{17}[b] (not quantified)
Hv3ox1	GA_{20}	GA_1 (25)
Hv3ox2	GA_{20}	GA_1 (5)
Hv2ox4	GA_{20}	GA_{29}[c] (90)
	GA_1	GA_8 (ND)
Hv2ox5	GA_{20}	GA_{29}[c] (ND)
	GA_1	GA_8 (65)

[a]Products were identified by their position on HPLC and GC-SIM spectra
[b]GA_{17} is derived from GA_{19} (Fig. 1) by the action of a 20ox
[c]GA_{29} is derived from GA_{20} (Fig. 1) by the action of a 2ox

ent-Kaurene oxidase

In barley, the HvKO1 probe detected at least three genes; the strongest hybridising band mapped to chromosome 7H and the two weaker bands to chromosomes 2H and 3H (Table 3). Based on the relative hybridisation intensity we conclude that *HvKO1* is located on chromosome 7H of barley. In wheat, gene family members have been identified on the long arm of chromosome groups 2 and 7 but due to lack of homology, insufficient resolution or a break in wheat-barley synteny no corresponding band was located on chromosome group 3. A search of the Genbank database using the HvKO1 protein sequence identified three closely related genes within 100 kb on the long arm of chromosome 6 in rice (R7, R8 and R9) (Fig. 2b). Because large segments of rice chromosome 6 have been reported to be syntenic to chromosome 7 of wheat (Gramene, http://www.gramene.org/cmap/viewer), *HvKO1* is likely to be the orthologue of one or more of the closely related sequences on chromosome 6 in rice (Fig. 4b). Based on sequence relatedness to HvKO1, the most likely candidate is the R7 sequence (81% identity).

ent-Kaurenoic acid oxidase

The HvKAO1 probe detected a single band from chromosome 7H in barley and three homoeologous bands on the short arm of group 7 in wheat (Table 3). A search of the Genbank database with the HvKAO1 protein sequence identified one closely related rice sequence (R10), which mapped to the short arm of chromosome 6 (Figs. 2b, 4b). Single-copy genes were located within syntenic regions on barley chromosome 7H and rice chromosome 6, suggesting that *HvKAO1* and *R10* may be orthologues.

20-oxidase

The Hv20ox1 probe identified a single band on chromosome 5H in barley and three homoeologous bands on the long arm of chromosome group 5 in wheat. A single band detected by the Hv20ox3 probe was mapped to chromosome 3H in barley and to the long arm of homoeologous group 3 in wheat. In rice, the *Os20ox1* and *Os20ox2* genes were previously positioned on chromosomes 3L and 1L, respectively. Synteny between barley chromosome 5H and rice chromosome 3 suggests that *Hv20ox1* and *Os20ox1* are orthologous genes; their protein sequences are also more closely related to each other than to other 20ox sequences (Figs. 2c, 4c). Similarly, known synteny between barley chromosome 3H and rice chromosome 1 suggests that

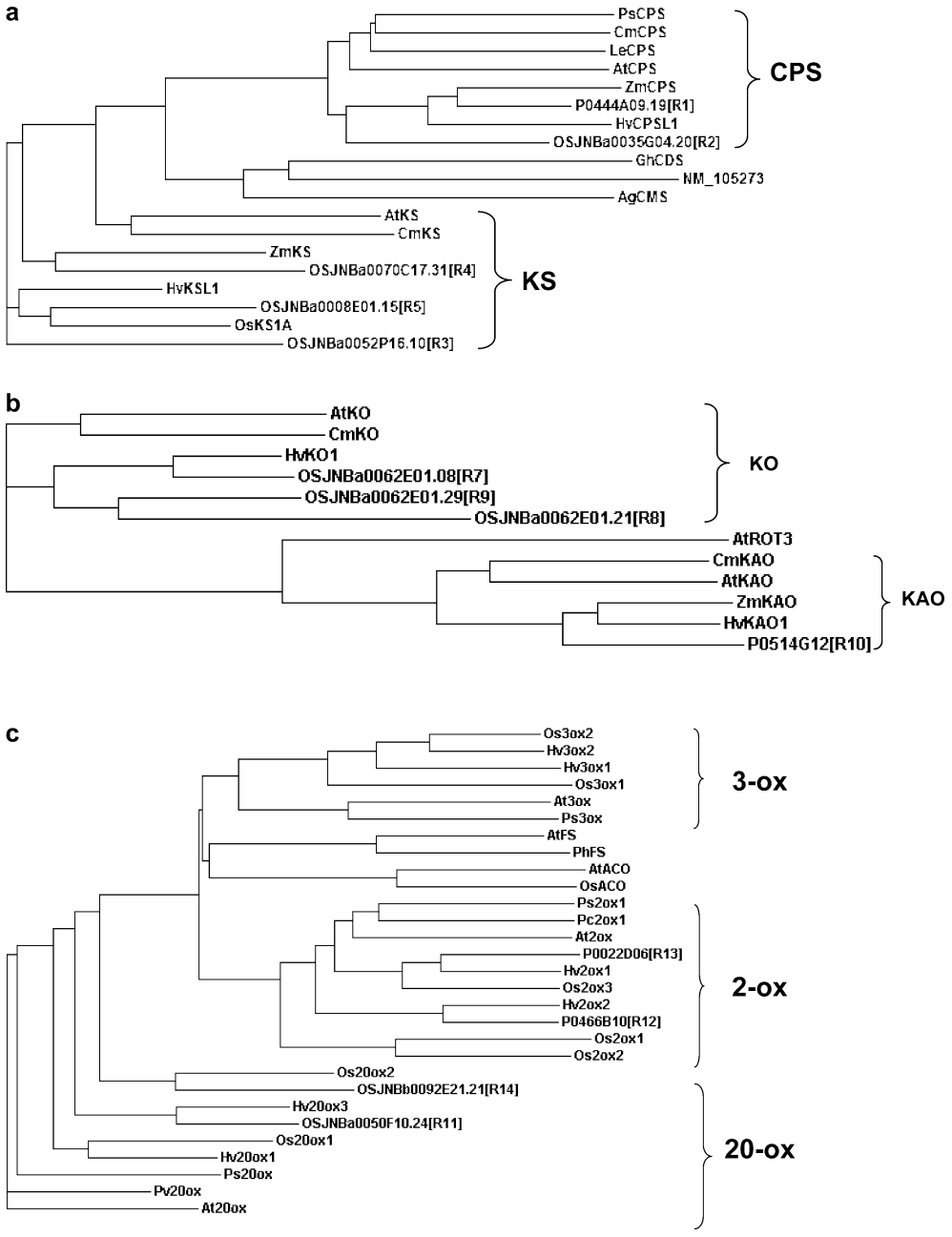

Fig. 2a–c Phylogram of GA biosynthetic and catabolic genes and related sequences. **a** Terpene cyclases, **b** cytochrome P450 monooxygenases, **c** dioxygenases. **a** Copalyl diphosphate synthases (*CPS*) from maize (*ZmCPS*, L37750), arabidopsis (*AtCPS*, U11034), pea (*PsCPS*, U63652), pumpkin (*CmCPS*, AF049905), tomato (*LeCPS*, AB015675), barley (*HvCPSL1*, AY551435) and related sequences from rice (*R1* and *R2*); kaurene synthases from pumpkin (*CmKS*, U43904), arabidopsis (*AtKS*, AF034744), maize (*ZmKS*, AF105149), barley (*HvKSL1*, AY551436) and related sequences from rice (*OsKS1A*, *R3*, *R4* and *R5*); closely related terpene cyclases that are not involved in GA metabolism include cadinene synthase from cotton (*GhCDS*, AF174294), terpene synthase from arabidopsis (*NM_105273*) and camphene synthase from *Abies grandis* (*AgCMS*, U87910). **b** Kaurene oxidases from arabidopsis (*AtKO*, AF318500), pumpkin (*CmKO*, AAG41776), barley (*HvKO1*, AY551434) and related sequences from rice (*R7*, *R8* and *R9*); kaurenoic acid oxidases from pumpkin (*CmKAO*, AF212991), arabidopsis (*AtKAO*, AF047719), maize (*ZmKAO*, U32579), barley (*HvKAO1*, AF326277) and a related sequence from rice (*R10*); closely related monooygenase gene from arabidopsis that is not involved in GA metabolism *rotundifolia3* (*AtROT3*, Q9 M066). **c** Gibberellin 20ox from rice (*Os20ox1*, U50333; *Os20ox2*, AY114310) including the related sequences (*R11* and *R14*), pea (*Ps20ox*, U58830), bean (*Pv20ox*, U70530), arabidopsis (*At20ox*, U20872), barley (*Hv20ox1*, AY551428; *Hv20ox3*, AY551429); gibberellin 3ox from rice (*Os3ox1*, AB054084; *Os3ox2*, AB056519), arabidopsis (*At3ox*, L37125), pea (*Ps3ox*, U93210), barley (*Hv3ox1*, AY551430; *Hv3ox2*, AY551431); gibberellin 2-oxidases from pea (*Ps2ox1*, AF101383), *Phaseolus coccineus* (*Pc2ox1*, AJ132438), arabidopsis (*At2ox*, AJ132435), barley (*Hv2ox4*, AY551432; *Hv2ox5*, AY551433), rice (*Os2ox1*, AB059416; *Os2ox2*, AB092484; *Os2ox3*, AB092485) including related sequences (*R12* and *R13*); closely related dioxygenases not involved in GA metabolism include flavonol synthases from arabidopsis (*AtFS*, BJ000494), petunia (*PhFS*, Z22543) and 1-aminocyclopropane-1-carboxylate oxidases from arabidopsis (*AtACO*, AY045876) and rice (*OsACO*, X85747). Rice sequences discussed in this study have been given a trivial nomenclature (*R1–R14*) and are listed as the predicted gene sequences (decimal point separates the clone descriptor from the predicted gene sequence) contained within a particular PAC/BAC clone

Table 3 Chromosomal locations of GA biosynthetic and catabolic gene markers in barley, wheat and rice

Enzyme	Barley genes	Chromosome location		Rice genes[a]	
		Barley	Wheat		
CPS	*HvCPSL1*	7H	7AL, 7BL, 7DL	R1 (2S)	R2 (9L)
		2HS (weak)	2AS, 2BS, 2DS	–	
KS	*HvKSL1*	2HL	2AL, 2BL, 2DL	OsKS1A, R4 (4L)	R3 (4S), R5 (2L)
	HvKSL2	2HL	2AL, 2B, 2DL	–	
KO	*HvKO1*	7H (strong)	7AL, 7BL, 7DL	R7, R8, R9 (6L)	
		2H (weak)	2AL, 2BL, 2DL	–	
		3H (weak)		–	
KAO	*HvKAO1*	7H	7AS, 4AL[b], 7DS	R10 (6S)	
20-Oxidase	*Hv20ox1*	5H	4AL[b], 5BL, 5DL	Os20ox1 (3L)	R11 (7S)
	Hv20ox3	3H	3AL, 3BL, 3DL	–	R14 (5L)
					Os20ox2 (1L)
3-Oxidase	*Hv3ox1*	2HL	2AL, 2BL, 2DL	–	Os3ox1 (5S)
	Hv3ox2	3HS	3AS, 3BS, 3DS	Os3ox2 (1S)	
2-Oxidase	*Hv2ox4*	1HL	1AL, 1BL, 1DL	R13 (5L)	Os2ox1 (5S)
	Hv2ox5	3H	3AS, 3BS, 3DS	R12 (1S)	Os2ox2 (1S)
					Os2ox3 (1L)

[a]Rice genes in the left-hand column are considered likely orthologues of the corresponding barley genes. Orthology remains unclear for rice sequences in the right-hand column. The trivial nomenclature (R1–R14) for rice sequences follows Fig. 2

[b]These chromosomal positions reflect ancestral translocations that were fixed in hexaploid wheat

Hv20ox3 may be orthologous to *Os20ox2*. However, Hv20ox3 has considerably higher sequence identity to a putative third rice 20ox—R11 (76% identity) located on chromosome 7—than it does to either Os20ox1 (58%) or Os20ox2 (53%). The syntenic relationship between barley chromosome 3HL and rice chromosome 7S remains unclear. An additional putative rice 20ox gene (R14) was identified on chromosome 5L that was closely related to *Os20ox2* (68% identity). No putative orthologous genes for *Os20ox2* or *Os20ox4* have yet been isolated from barley.

3-oxidase

The Hv3ox1 probe was mapped to the long arm of chromosome 2H in barley and to homoeologous group 2 in wheat, whereas Hv3ox2 markers were located on the short arm of chromosome 3H of barley and group 3 of wheat (Table 3). Because Hv3ox1 and Hv3ox2 probes shared 78% sequence identity, and therefore cross-hybridised, genes were assigned to specific chromosomal locations on the basis of relative hybridisation intensity. In rice, *Os3ox1* and *Os3ox2* have been isolated and mapped to chromosome 5S and 1S, respectively (Itoh et al. 2001). Chromosomal regions on 3HS of barley are syntenic to regions on 1S of rice, suggesting that the *Hv3ox2* and *Os3ox2* genes are orthologues (Fig. 4c). This result is supported by the sequence alignment, which showed that both genes are more closely related to each other than to other 3ox genes (Fig. 2c). In contrast, the relationship between *Hv3ox1* and *Os3ox1* is less clear; synteny between their respective chromosomal regions is not known, and Hv3ox1 shares slightly greater sequence identity with Os3ox2 (62% identity) than with Os3ox1 (56% identity) as was observed for Hv20ox3 and the corresponding rice genes (Fig. 2c). No other 3ox sequences were identified in the rice genome.

2-oxidase

The Hv2ox4 probe hybridised to several fragments in barley and wheat with the strongest mapping to the long arm of chromosome 1H in barley and chromosome group 1L in wheat. The Hv2ox5 probe also gave several bands with the strongest hybridising bands positioned on the short arm of chromosome 3H in barley and on the corresponding wheat chromosomes. In rice, three closely related 2ox genes (*Os2ox1*, *Os2ox2* and *Os2ox3*) have been characterised and mapped to rice chromosome 1 and 5 (Fig. 4c) (Sakai et al. 2003; Sakamoto et al. 2001). Searches of the rice genome with previously isolated 2ox sequences identified two additional sequences, R12 and R13, which mapped to the short arm of chromosome 1 and the long arm of chromosome 5, respectively (Fig. 4c). Alignments of amino acid sequences and comparative mapping results suggest that *Hv2ox4* is an orthologue of *R13* and that *Hv2ox5* is an orthologue of *R12*.

Discussion

The isolation of barley genes that encode enzymes involved in GA biosynthesis or catabolism is reported. Together, these 11 genes potentially account for the six enzymes involved in the core GA biosynthetic pathway and for the first major catabolic enzyme. Two of the genes (*HvKO1*, *HvKAO1*) encode biosynthetic enzymes that are important for GA biosynthesis in vivo, since GA-deficient mutants of barley show specific alterations in these particular sequences. Six dioxygenase genes (*Hv20ox1*, *Hv20ox3*, *Hv3ox1*, *Hv3ox2*, *Hv2ox4* and *Hv2ox5*) were

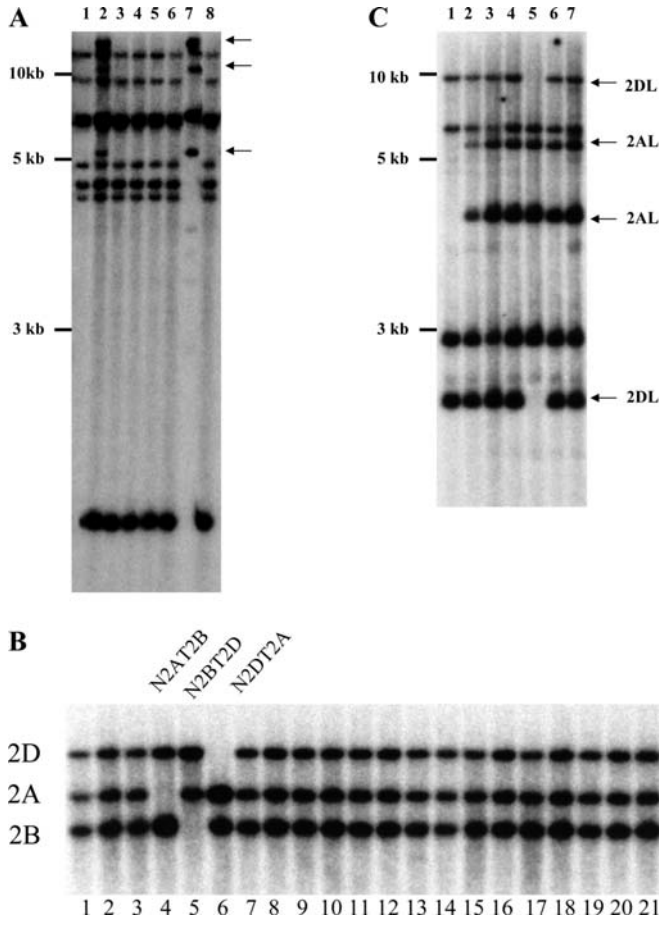

Fig. 3a–c DNA blot analysis of the *HvKSL2* gene. **a** Autoradiogram of a DNA blot containing digested genomic DNA (*Eco*RI) of Chinese Spring wheat/Betzes barley chromosome addition lines. *Lanes 1–6* wheat plus barley chromosomes 1, 2, 3, 4, 6 and 7, respectively, *lane 7* digested genomic DNA of Betzes barley, *lane 8* digested genomic DNA of Chinese Spring wheat. *Arrows* show three bands that are unique to barley and which are found only in the wheat line containing barley chromosome 2. We therefore conclude that bands are present on chromosome 2H. **b** Autoradiogram of blot containing *Hin*dIII digested genomic DNA of nullitetra-disomic Chinese Spring aneuploid stocks. *Lanes 1–3* DNA of nullitetra lines for chromosome group 1, *lanes 4–6* DNA of nullitetra lines for chromosome group 2, *lanes 7–9* for chromosome group 3, *lanes 10–12* are for chromosome group 4, *lanes 13–15* are for chromosome group 5, *lanes 16–18* are for chromosome group 6, *lanes 19–21* are for chromosome group 7. *N2AT2B* Nullisomic 2A tetrasomic 2B, *N2BT2D* nullisomic 2B tetrasomic 2D, *N2DT2A* nullisomic 2D tetrasomic 2A. Polymorphism detected between homoeologous *HvKSL2* gene members enabled individual gene members to be assigned to chromosome 2A, 2B and 2D. **c** Autoradiogram of a blot containing digested genomic DNA (*Dra*I) of ditelosomic Chinese Spring aneuploid stocks for chromosome group 2. *Lanes 1–6* the following lines: ditelosomic 2AS, ditelosomic 2AL, ditelosomic 2BS, ditelosomic 2BL, ditelosomic 2DS, ditelosomic 2DL, *lane 7* digested genomic DNA of euploid Chinese Spring wheat. *Arrows* indicate hybridisation bands that are located on the long arm of chromosome 2A and long arm of chromosome 2D. The predicted gene member located on chromosome 2BL is not polymorphic in relation to the other homoeologous chromosomes

shown to have in vitro activity. The remaining clones (*HvCPSL1*, *HvKSL1* and *HvKSL2*) are not yet directly linked to GA metabolism in vivo, but they show high sequence relatedness to corresponding genes of demonstrated function in other species. The gene responsible for 13-oxidation of GAs has not yet been characterised, although it could correspond to one of the rice monooxygenase or dioxygenase sequences reported here (see below). In four cases we isolated clones for distinct isozyme pairs, suggesting that there is a degree of redundancy in the metabolic pathway and/or that different parts of the plant utilise different isozymes. In some cases there were large differences between isozyme pairs in their in vitro substrate specificity. For instance, the Hv2ox4 enzyme failed to catabolise GA_1 to GA_8 but did convert GA_{20} to GA_{29}, thereby potentially diverting the GA_{20} precursor from oxidation to the bioactive GA_1. The substrate specificity of Hv2ox5 was reversed, suggesting that it is one of the catabolic enzymes of GA_1 in barley.

We used barley genes and other cereal genes involved in GA metabolism to search rice genome databases for closely related sequences. The search results were combined with previously published reports and database entries which identified coding sequences related to most GA biosynthetic and catabolic enzymes in rice. Only the most closely related sequences were considered, although it is still not certain that all of these genes are functional. Multiple-sequence alignments confirmed that the rice sequences were members of gene families and that the copy number within each group ranged from a single-copy gene, *R10* (similar to the *ent*-KAO gene), to at least five gene members for the 2ox gene family. Gene duplications within small physical intervals were probably responsible for two closely related *ent*-KS genes occurring on chromosome 4 and for at least three sequences closely related to *ent*-kaurene oxidase genes on chromosome 6. A larger scale event involving the duplication of a chromosomal segment on chromosome 1 and 5 may have produced two segments carrying related 2-oxidase and 3-oxidase genes (Fig. 4c).

Previous studies using DNA probes of unknown function revealed moderate levels of conservation in gene content and gene order between rice and Triticeae chromosomes (Gale and Devos 1998). The newly released rice genome sequence and the mapping of probes with known function have rapidly expanded the comparative map information for cereal genomes (Gramene). In the present study, map locations of barley genes encoding GA biosynthetic and catabolic enzymes corresponded to chromosome arm positions identified in wheat, reflecting the high level of synteny previously reported between barley and wheat chromosomes. For each barley gene, with the exception of *Hv20ox3* and *Hv3ox1*, it was also possible to identify closely related sequences in syntenic regions of rice. In most cases, genes in syntenic locations also shared the highest level of sequence relatedness compared to other sequences from the same gene family. Our limited knowledge of synteny, in particular a poor understanding of micro-synteny that exists between small

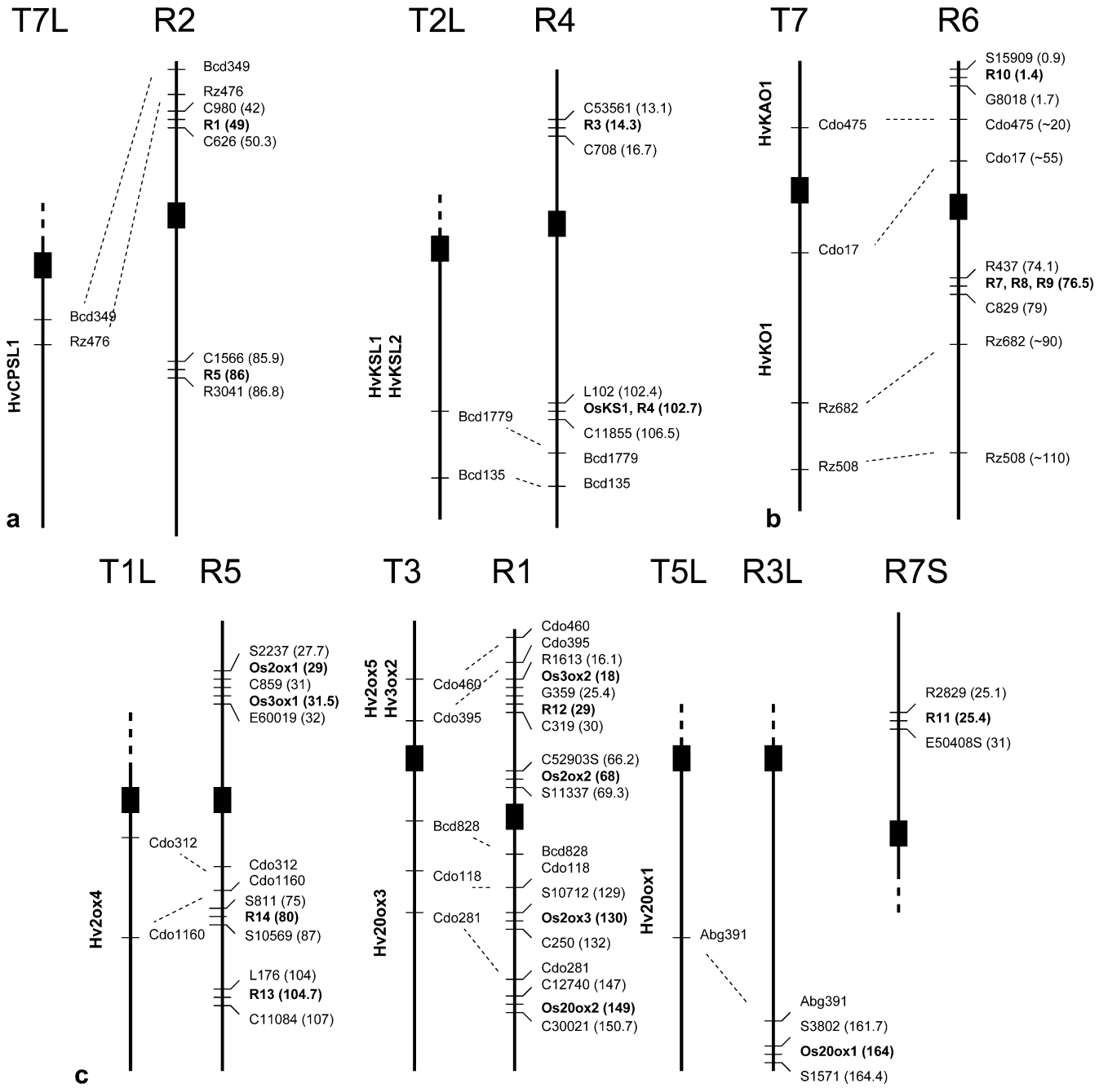

Fig. 4a–c Comparative mapping results between Triticeae (*T*) and rice (*R*) chromosomes. Barley genes were mapped at the chromosome arm level, and syntenic regions were determined using the COMPARATIVEMAP VIEWER on Gramene (http://www.gramene.org/cmap/viewer). Rice genes identified in this study use the trivial nomenclature *R1–R14* (see Fig. 2 for details). Tightly linked markers and genetic distance estimates were included (shown in centiMorgans, *in parenthesis*) according to the high-density Japanese Rice Genome Program (JRGP) map shown on Gramene. Comparative mapping results are shown for terpene cyclases (**a**), cytochrome P450 monooxygenases (**b**) and dioxygenases (**c**)

physical intervals, may in some cases have prevented us from identifying orthologous genes. For example, sequence comparisons of 20ox genes suggested that *Hv20ox3* on barley chromosome 3H and *R11* on rice chromosome 7 may be orthologues, but no colinear markers have previously been reported for these chromosomal regions. It is possible that either *R11* or *Hv20ox3* has moved to a non-syntenic region in a small-scale translocation event. The rice *adh1* gene was predicted to have undergone a similar translocation event since this region exhibits no colinearity with the maize or sorghum *adh1* region beyond the gene itself (Bennetzen 2000).

This study has provided new information on the gene families and map positions of GA biosynthetic and catabolic genes in cereals. Our collection of barley cDNA clones for GA metabolic enzymes is still

incomplete, but the putative genes identified from the rice genome provides a useful framework for identifying the corresponding barley sequences. This information will be used as a first step to determine the extent to which dwarfism in barley and wheat is caused by defects in GA metabolism.

Note added in proof After acceptance of our manuscript Sakamoto et al (2004) published an analysis of GA biosynthetic genes in rice. They identified functional gene sequences for rice CPS1, KS1, KO1 and KAO1, since mutations in these sequences resulted in dwarfism and GA deficiency. They also reported additional sequences for rice GA dioxygenases. Overall their results are in very close agreement (see below) with the rice genes predicted in our study (Figs 2A,B,C). The only difference is our prediction of a fifth rice 2-oxidase (sequence R12), presumably orthologous to the functional Hv2ox5, which was not predicted by Sakamoto et al (2004).

Gene	Predicted rice sequence		
	This study		Sakomoto et al. 2004
HvCPSL1	R1	=	OsCPS1
HvKSL1	OsKS1A	=	OsKS1
HvKAO1	R10	=	OsKAO1
Hv20ox3	R11	=	OsGA20ox3
Hv2ox4	R13	=	OsGA2ox4
Hv2ox5	R12		Not detected

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