

H. Shinozuka · H. Hisano · R. C. Ponting
N. O. I. Cogan · E. S. Jones · J. W. Forster
T. Yamada

Molecular cloning and genetic mapping of perennial ryegrass casein protein kinase 2 α -subunit genes

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Abstract The α -subunit of the casein protein kinase CK2 has been implicated in both light-regulated and circadian rhythm-controlled plant gene expression, including control of the flowering time. Two putative CK2 α genes of perennial ryegrass (*Lolium perenne* L.) have been obtained from a cDNA library constructed with mRNA isolated from cold-acclimated crown tissue. The genomic organisation of the two genes was determined by Southern hybridisation analysis. Primer designs to the *Lpck2a-1* and *Lpck2a-2* cDNA sequences permitted the amplification of genomic products containing large intron sequences. Amplicon sequence analysis detected single nucleotide polymorphisms (SNPs) within the p150/112 reference mapping population. Validated SNPs, within diagnostic restriction enzyme sites, were used to design cleaved amplified polymorphic sequence (CAPS) assays. The *Lpck2a-1* CAPS marker was assigned to perennial ryegrass linkage group (LG) 4 and the *Lpck2a-2* CAPS marker was assigned to LG2. The location of the *Lpck2a-1* gene locus supports the previ-

ous conclusion of conserved synteny between perennial ryegrass LG4, the Triticeae homoeologous group 5L chromosomes and the corresponding segment of rice chromosome 3. Allelic variation at the *Lpck2a-1* and *Lpck2a-2* gene loci was correlated with phenotypic variation for heading date and winter survival, respectively. SNP polymorphism may be used for the further study of the role of CK2 α genes in the initiation of reproductive development and winter hardiness in grasses.

Keywords Perennial ryegrass · Casein kinase 2 α -subunit · Single nucleotide polymorphism · Cleaved amplified polymorphic sequence · Heading date · Conserved synteny

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H. Shinozuka · H. Hisano · T. Yamada (✉)
Field Science Center for Northern Biosphere,
Hokkaido University, Kita 11, Nishi 10,
Kita, Sapporo 060-0811, Japan
E-mail: yamada@fsc.hokudai.ac.jp
Tel.: +81-11-7063644
Fax: +81-11-7063644

R. C. Ponting · N. O. I. Cogan · E. S. Jones · J. W. Forster
Primary Industries Research Victoria, Plant Biotechnology Centre
and Molecular Plant Breeding Cooperative Research Centre,
La Trobe University, Bundoora, Victoria 3086, Australia

E. S. Jones
Crop Genetics Research and Development,
Pioneer Hi-Bred International, 7300 NW 62nd Avenue,
Johnston, IO 50131-1004, USA

T. Yamada
National Agricultural Research Center for Hokkaido Region,
National Agriculture and Bio-oriented Research Organization,
Hitsujiigaoka, Sapporo 062-8555, Japan

Introduction

Casein protein kinase 2 (CK2) is a multifunctional serine–threonine dependent protein kinase that is ubiquitously distributed in eukaryotes and is involved in signal transduction events that are relevant to cell proliferation (Litchfield and Lüscher 1993). CK2 possesses a characteristic tetrameric structure ($\alpha_2\beta_2$), composed of two catalytic α -subunits and two regulatory β -subunits (Pinna 1990; Tuazon and Traugh 1991), and is capable of phosphorylating a wide variety of substrates, including transcription factors (Meggio and Pinna 2003). The analysis of two *Arabidopsis thaliana* CK2 α -subunit genes (Mizoguchi et al. 1993) suggested that CK2 may function as one of several quantitative negative effectors of light-regulated gene expression (Lee et al. 1999), and is necessary for the normal functioning of the *A. thaliana* circadian clock (Daniel et al. 2004). In rice (*Oryza sativa* L.), the major chromosome 3-located *Hd6* heading date QTL is associated with the inhibition of flowering under long day conditions (Yamamoto et al. 2000), and encodes an α -subunit of CK2 (Takahashi et al. 2001). The variant allele of the CK2 α gene, in the *japonica* rice variety Nipponbare, encodes a putatively non-functional protein with a premature stop codon, while

the corresponding functional allele, in the *indica* rice variety Kasalath, is associated with an increase in days-to-heading when compared to reference varieties (Takahashi et al. 2001).

The genetic control of flowering time variation has been extensively studied in the Triticeae tribe of small-grained cereals (wheat, barley, rye and their relatives). Conserved genomic locations for genes involved in processes such as vernalisation and photoperiodic induction have been identified between species by comparative genetic studies (Dubcovsky et al. 1998). Vernalisation requirement and photoperiod sensitivity are also intimately related to traits such as low-temperature tolerance in grass and cereal species (Limin and Fowler 2002). An improved knowledge of the genetics of winter hardiness would have a significant impact on world agriculture, as low temperature-related stresses limit the productivity of many plant species. In barley (*Hordeum vulgare* L.), the long arm of chromosome 5H contains QTL and major gene loci for vernalisation response, photoperiod response and cold tolerance (Pan et al. 1994; Cattivelli et al. 2002), as well as members of the temperature stress-related Cold-regulated (COR) and C-repeat binding factor (CBF) gene families (Francia et al. 2004). Putative orthologous sequences are observed on the long arms of the group-5 homeologous chromosomes of bread wheat (*Triticum aestivum* L.), including the *Vrn1* major vernalisation response loci (Dubcovsky et al. 1998; Cattivelli et al. 2002). Comparative genetic mapping studies between rice and wheat, based on the colinearity of common restriction fragment length polymorphism (RFLP) markers (Kato et al. 1999), have revealed that the rice *Hd6* locus region on chromosome 3 is syntenic with the wheat *VrnA1* region on chromosome 5AL. Genetic mapping, using a wheat CK2 α gene (*tck2a*) probe, detected a genetic locus closely linked (by 1.1 cM) to *VrnA1* (Kato et al. 2002).

Perennial ryegrass is an important temperate pasture grass species. Selection for variation in the time of reproductive maturity is of importance for ryegrass breeders in order to mitigate the effects of seasonal changes in herbage quality. In addition, the cultivation of this species in the colder regions of the northern biosphere is limited by tolerance to freezing stress and associated damage by pathogens such as snow mould. Enhancement of winter hardiness is, consequently, an important breeding objective (Wilkins and Humphreys 2003). Significant correlation was observed between the heading date and freezing tolerance, such that early heading accessions tend to be less tolerant of freezing than late heading accessions (Humphreys and Eagles 1988). Despite these physiological studies, relatively little is known about the genetic control of flowering time and winter hardiness in perennial ryegrass.

The construction of molecular marker-based genetic linkage maps, based on one-way pseudo-testcross, two-way pseudo-testcross and F₂ populations (Jones et al. 2002a, b; Faville et al. 2004; Armstead et al. 2002; Forster et al. 2004), has provided the basis for

trait-dissection activities in perennial ryegrass. In addition to studies of disease resistance (Dumsday et al. 2003), flowering time variation (Armstead et al. 2004; Yamada et al. 2004; Jensen et al. 2005), morphogenetic traits (Yamada et al. 2004) and herbage quality (Cogan et al. 2005), a QTL for electrical conductivity corresponding to frost tolerance has been located on LG4, close to a heading date QTL (Yamada et al. 2004). Comparative genetic mapping studies have revealed that the upper part of perennial ryegrass LG4 contains a region of conserved synteny with the rice chromosome 3 and the long arms of the Triticeae homeologous group-5 chromosomes (Jones et al. 2002a; Yamada et al. 2004; Sim et al. 2005). The genomic regions in which the CK2 α genes of pasture grasses are located are, therefore, of considerable interest for the genetic analysis of both flowering time variation and winter hardiness.

In this study, we have identified two CK2 α gene sequences (*Lpck2a-1* and *Lpck2a-2*), from a perennial ryegrass cDNA library, and assigned them to chromosomal locations using the enhanced reference genetic map constructed through the activities of the International *Lolium* Genome Initiative (ILGI: Forster et al. 2001). This activity has identified a region of putative conserved synteny with the wheat *VrnA1* region and provides further support for chromosomal translocations during the evolution of the Triticeae and Poaceae lineages of the Poaceae. In addition, allelic variation at the *Lpck2a* gene loci was significantly associated with phenotypic variation for days-to-heading and winter survival, respectively, in the reference genetic mapping population.

Materials and methods

Isolation of perennial ryegrass CK2 α cDNA clones

Perennial ryegrass [cultivar (cv.) Aberystwyth S23] plants were grown in a controlled climate chamber (16 h day length, 22°C/18°C day/night) for 30 days and were cold-acclimated (8 h day length, 6°C/2°C day/night) for 2 weeks. A cDNA library was constructed from mRNA extracted from the crown tissues of cold-acclimated plants, using a ZAP Express[®] cDNA Synthesis Kit and a ZAP Express cDNA Gigapack[®] III Gold Cloning Kit (Stratagene) according to the manufacturer's instructions. A CK2 α gene-specific probe was obtained by PCR amplification from this cDNA library, using the primer pair combination *tck2aF* (5'-ATCATGAGCTT-CGAAACTC-3') and *tck2aR* (5'-TCACGTGC-GGTGAGCCTATC-3') designed on the basis of the DNA sequence of the wheat CK2 α cDNA (GenBank Accession No. AB052133: Kato et al. 2002).

The amplified PCR fragments were labelled and used to screen the cDNA library by plaque hybridisation, using the Gene Images AlkPhos Direct Labelling and Detection Kit (GE Healthcare). A total of ca. 3×10⁴ recombinant plaques were screened and positive plaques

were isolated. After two cycles of plaque purification, *in vivo* excisions of the pBK-CMV phagemid vector were performed in the *E. coli* XL0LR strain. The nucleotide sequences of the inserts were determined using the CEQ™ 8000 Genetic Analysis System (Beckman Coulter).

Molecular phylogenetics analysis

CK2 α -subunit protein sequences were obtained using BLAST applications at the National Centre of Biotechnology Information (NCBI) web site (<http://www.ncbi.nlm.nih.gov/>). Multiple sequence alignments and phylogenetic trees were constructed by the neighbour joining (NJ) method, using the CLUSTALW program in Genome Net (<http://www.genome.jp/>). 'BLOSUM' was chosen as the weight matrix for amino acid sequence alignment. Graphical representations of phylogenetic trees were produced with CLUSTALW and were manually edited.

Southern hybridisation analysis

Genomic DNA extracted from the young leaves of an individual plant from cv. Aberystwyth S23, using the modified CTAB method (Murray and Thompson 1980), was digested with a number of restriction enzymes (*Dra*I, *Eco*RI, *Hind*III, *Sac*I and *Xba*I). The digested DNA was transferred to Hybond N or N⁺ nylon membranes (GE Healthcare), prior to Southern hybridisation, using the perennial ryegrass CK2 α cDNA clones as probes. The hybridisation assay was performed using the Gene Images AlkPhos Direct Labelling Detection Kit (GE Healthcare).

Plant material

The p150/112 reference genetic mapping population (Bert et al. 1999; Jones et al. 2002a, b; Yamada et al. 2004) was used for genetic analysis. Phenotypic analyses of the heading date, winter survival and electrical conductivity traits were performed as described by Yamada et al. (2004).

SNP detection and validation

The primer pairs were designed to amplify genomic regions specific to each CK2 α gene. For the *Lpck2a-1* gene, the initial primer design was directed to exonic sequences (*Lpck2a-1EF*: 5'-CAGCCAGCTTCGTAT-TTTCG-3'; *Lpck2a-1ER*: 5'-CTGGGAGTCTTCGAA-TGTTG-3'), and the subsequent nested design was directed to intron sequences (*Lpck2a-1IF*: 5'-TGAT-CACTGTCAATTGCAGC-3'; *Lpck2a-1IR*: 5'-ACA-ATGGAAGGTCTCCATCA-3'). For the *Lpck2a-2* gene, the primers designed from exon sequences were

designated *Lpck2a-2EF* (5'-ATAGACTGGGGCC-TTGCTGA-3') and *Lpck2a-2ER* (5'-GGTGATCATA-GCGCAGAAGC-3'). The conditions for locus-specific PCR amplification were: one time PCR buffer, 1.5 mM MgCl₂, 0.2 mM each dNTP, 0.25 nM each primer, and 0.25 units Hotstar polymerase (Qiagen) per 20 μ l reaction. The cycling conditions consisted of an initial polymerase activation step at 94°C for 15 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, ending with 72°C for 7 min and an indefinite period at 4°C. PCR reactions were performed using ABI9700 thermocyclers (Applied Biosystems).

Amplified fragments were cloned using the TOPO TA for Sequencing Cloning® vector (Invitrogen), followed by transformation into One Shot® TOP10 *E. coli* recipient cells, all according to the manufacturer's instructions. Transformants were selected on an LB medium containing carbenicillin as the selective antibiotic, and the recombinants were identified by colony PCR as described above. The plasmid template was prepared for DNA sequencing, using the TempliPhi™ procedure (GE Healthcare) according to the manufacturer's instructions. DNA sequencing was performed using DYEnamic ET dye terminator cycle sequencing reagents (GE Healthcare), with 2 μ l of amplified product and 500 nM T3 or T7 universal primers in a 10 μ l reaction. Capillary electrophoresis was performed using MegaBACE 1,000 or 4,000 capillary electrophoresis platforms (GE Healthcare).

DNA sequence trace files were analysed using the Sequencher™ Version 4.1.4 for Windows application (Gene Codes Corporation) to assemble contigs and identify putative SNPs. Primers to sequences directly adjacent to the variant bases were designed for the single nucleotide primer extension (SNUPE) assay, which was performed using the GE Healthcare genotyping kit, according to the manufacturer's instructions.

SNP genotyping was performed using the MegaBACE 1,000 capillary electrophoresis platform (GE Healthcare), with the following run parameters: injection at 12 kV for 20 s, with an interval time of 90 s at 9 kV and separation conditions of 6 kV for 50 min. SNP alleles were scored with SNP Profiler (GE Healthcare).

Cleaved amplified polymorphic sequence (CAPS) assay

Genomic DNA was extracted from perennial ryegrass leaf tissue using the modified CTAB method (Murray and Thompson 1980). The PCR amplicons generated, using the *Lpck2a-1IF/R* primer pair, were digested with the restriction enzyme *Pvu*II, while the PCR amplicons generated using the *Lpck2a-2EF/R* primer pair were digested with the restriction enzyme *Sac*I. The digested fragments were separated in 1% (w/v) horizontal agarose gels using one time Tris-Borate-EDTA (TBE) buffer. The DNA fragments were visualized and photographed under UV light, following staining with 0.5 μ g/ml ethidium bromide.

CAPS markers were mapped within the context of a framework set of genetic markers from the p150/112-based reference map (Jones et al. 2002a), including the majority of the heterologous RFLP loci and the perennial ryegrass SSR locus data (Jones et al. 2002b), using the MAPMAKER 3.0 application (Lander et al. 1987). In order to permit correlation of the map data with the published study of Yamada et al. (2004), the locations of *xlpc2a* loci were interpolated onto the map order and appropriate interlocus distances, as described by Jones et al. (2002b).

Analysis of association between CAPS locus-specific genotype and phenotypic variation

The effects of allelic variation at the CAPS locus-specific markers on the heading date, winter survival and electrical conductivity (freezing tolerance) traits, described by Yamada et al. (2004), were analysed using single marker-based analysis of variance with generalised linear modelling (GLM) in the SAS software package (SAS Institute Inc.). Interactions between the two gene loci were analysed by placing both markers into the model as independent variables. Differences between means were analysed using Tukey's studentised range (HSD) test.

Comparative genomics analysis

A comparative genomics analysis of perennial ryegrass CK2 α genes was performed using the wEST SQL database in the GrainGenes resource. The nucleotide sequences were used for BLASTN and TBLASTX analysis in the GrainGenes BLAST page, with the search restricted to ESTs that have been assigned to wheat deletion bins (Qi et al. 2003) (<http://wheat.pw.usda.gov/wEST/blast/>). The highest matching ESTs were then used to detect the relevant deletion bins, using the Mapped Loci query function in wEST (http://wheat.pw.usda.gov/cgi-bin/westsql/map_locus.cgi).

Results

Isolation and characterisation of perennial ryegrass CK2 α cDNA clones

Two cycles of hybridisation-based screening of the perennial ryegrass cDNA library led to the identification of ten positive plaques. Following sub-cloning and DNA sequence analysis, sequence similarity searches, using the BLASTN application, demonstrated that two distinct classes of cDNAs were included in the positive plaques; both encoding proteins with high similarity to known CK2 α -subunits. The two cDNAs were designated *Lpck2a-1* (GenBank Accession No. AB213316) and *Lpck2a-2* (GenBank Accession No. AB213317). The *Lpck2a-1* cDNA class was most similar at the sequence

level (89% identity) to the rice *OsCK2a* gene (GenBank Accession No. AB036788). The longest cDNA of this class consists of 1,629 bp, including 26 bp of poly(A) tail, and contains an open reading frame (ORF) encoding a 381 amino acid protein. The *Lpck2a-2* cDNA class was most similar, at the sequence level (93% identity), to the wheat *tck2a* gene (GenBank Accession No. AB052133). The longest cDNA of this class consists of 1,472 bp, including 19 bp of poly(A) tail, and contains an ORF encoding a 333 amino acid protein. The two perennial ryegrass CK2 α genes shared 73% nucleotide identity, lower than the equivalent values observed for the comparison of *Lpck2a-1* with *OsCK2a* (89%) and *Lpck2a-2* with *tck2a* (93%), and indicating sequence divergence between the two perennial ryegrass gene classes prior to speciation. The *Lpck2a-2* cDNA class also showed a high level of similarity (90%) with the second rice CK2 α gene variant (represented by GenBank Accession No. NM94133), although sequence identity in comparison to *OsCK2a* was still high (88%).

The alignment of the deduced amino acid sequences of the perennial ryegrass CK2 α genes and related sequences from public databases was performed (Fig. 1a). The *Lpck2a-1* and *Lpck2a-2* primary protein sequences showed 91.0 and 92.5% identity, respectively, with an *A. thaliana* CK2 α -subunit protein. Two specific biologically significant catalytic domains, for ATP binding and the serine-threonine protein kinase active site, were highly conserved in the two perennial ryegrass CK2 α genes. Phylogenetic relationships between multiple CK2 α protein sequences were determined (Fig. 1b). Essentially, similar relationships were obtained through a comparison of gene sequences (data not shown). The *Lpck2a-1* gene product is located in a sub-cluster with the rice *OsCK2a* protein, while the *Lpck2a-2* gene product is located in a separate sub-cluster, with the *tck2a* and NM94133-encoded proteins. This result suggests that the two perennial ryegrass genes represent distinct sub-families. A third closely related sub-cluster contained sequences only from maize (*Zea mays* L.).

Southern hybridisation analysis, using both CK2 α cDNAs as probes, revealed multiple banding patterns with five different restriction enzymes. The *Lpck2a-1* probe detected an average of 2.2 bands, while the *Lpck2a-2* probe detected an average of 3.2 bands. For each enzyme, apart from *SacI*, common bands were identified between the two hybridisations (data not shown). This result is consistent with the observed sequence similarity between the two genes and the presence of a small multigene family of perennial ryegrass CK2 α genes, containing at least two sub-families.

SNP detection and validation

The cDNA sequences were used to design genomic amplicons. Preliminary sequencing of the 2.2 kb amplicon, generated by the exon-located primers for *Lpck2a-1*, identified a large intron from which nested

Fig. 1 a Alignment of deduced amino acid sequences for CK2 α genes. The serine-threonine protein kinase site (*grey shade*) and the ATP-binding site (*box*) are indicated. Asterisks represent stop codons. The species of origin is represented before the sequence and the relevant GenBank accession number is indicated following the sequence. The *Lpck2a-1* gene is capable of encoding a putative leader peptide, which is included in the alignment structure. **b** Phylogenetic relationships between 18 CK2 α -subunit proteins obtained by the NJ method in CLUSTALW. Species of origin and accession numbers are indicated. The accession number of each gene is represented following origin. The *black oval* indicates putative orthologues of *Lpck2a-1*, and the *grey oval* indicates putative orthologues of *Lpck2a-2*. In addition to the species included in **a**, the following taxa were included: *Nicotiana tabacum* (tobacco); *Beta vulgaris* (sugar beet); *Lilium davidii* (lily)

a

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Lpck2a-1 MSAAPPSSKRPASVFVSTAAAVLIAALASSLIALSQRQAPPAALRPIMSKARVYTDVNVVRPKEYWDYEAALTVQWGEQDDYEV
O.sativa MSKARVYADVNVLRPKEYWDYEAALTVQWGEQDDYEV
Lpck2a-2 MSKAKVYADVNVVRPKEYWDYEAALAVQWGEQDDYEV
T.aestivum MSKARVYADVNVVRPKEYWDYEAALAVQWGEQDDYEV
O.sativa MSKARVYTDVNVLRPKEYWDYEAALTVQWGEQDDYEV
Z.mays MSKAKVYTDVNVLRPKEYWDYEAALTVQWGEQDDYEV
A.thaliana MSKARVYTEVNVIRPKDYWDYESLIVQWGEQDDYEV

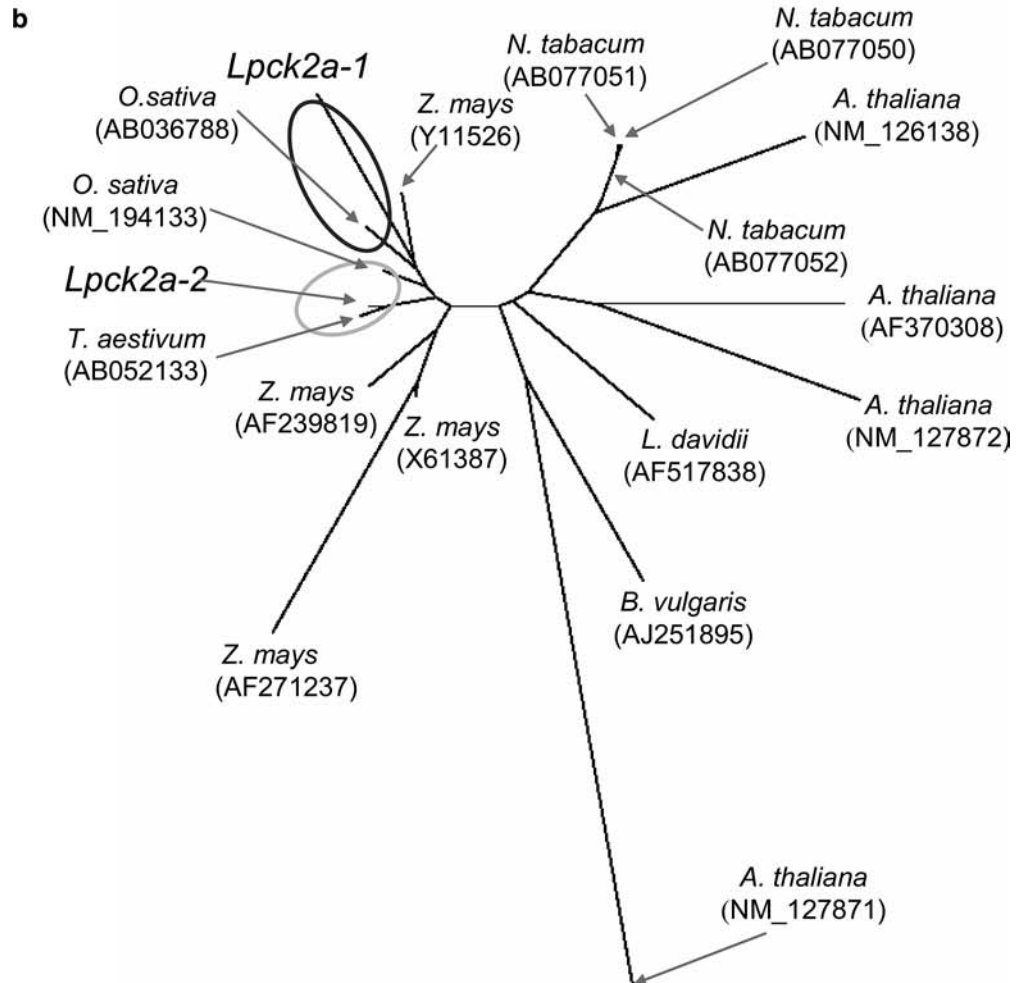
Lpck2a-1 VRKVGGRGKYSEVFEGINVTNSEKCVIKILKPVKKKKIKREIKILQNLGGPNIIKLLDIDVRDQHSKTPSLIFEYVNNSTDFKVLV
O.sativa VRKVGGRGKYSEVFEGINVNNEKCIKILKPVKKKKIKREIKILQNLGGPNIVKLLDIDVRDQHSKTPSLIFEYVNNSTDFKVLV
Lpck2a-2 VRKVGGRGKYSEVFEGINVNNEKCVIKILKPVKKKKIKREIKILQNLGGPNIVKLLDIDVRDQHSKTPSLIFEYVNNSTDFKVLV
T.aestivum VRKVGGRGKYSEVFEGINVNNEKCIKILKPVKKKKIKREIKILQNLGGPNIVKLLDIDVRDQHSKTPSLIFEYVNNSTDFKVLV
O.sativa VRKVGGRGKYSEVFEGINVNNEKCIKILKPVKKKKIKREIKILQNLGGPNIVKLLDIDVRDQHSKTPSLIFEYVNNSTDFKVLV
Z.mays VRKVGGRGKYSEVFEGINVNNEKCIKILKPVKKKKIKREIKILQNLGGPNIVKLLDIDVRDQHSKTPSLIFEYVNNSTDFKVLV
A.thaliana VRKVGGRGKYSEVFEGINVNSEKCIKILKPVKKKKIKREIKILQNLGGPNIVKLLDIDVRDQHSKTPSLIFEYVNNSTDFKVLV

Lpck2a-1 PTLTDYDIRYYIYELLKALDYCHSQGIMHRDVKPHNVIMIDHELRLRLIDWGLAEFYHPGKEYNVRVASRYFKGPELLVDLQDY
O.sativa PTLTDYDIRYYIYELLKALDYCHSQGIMHRDVKPHNVIMIDHELRLRLIDWGLAEFYHPGKEYNVRVASRYFKGPELLVDLQDY
Lpck2a-2 PTLTDYDIRYYIYELLKALDYCHSQGIMHRDVKPHNVIMIDHELRLRLIDWGLAEFYHPGKEYNVRVASRYFKGPELLVDLQDY
T.aestivum PTLTDYDIRYYIYELLKALDYCHSQGIMHRDVKPHNVIMIDHELRLRLIDWGLAEFYHPGKEYNVRVASRYFKGPELLVDLQDY
O.sativa PTLTDYDIRYYIYELLKALDYCHSQGIMHRDVKPHNVIMIDHELRLRLIDWGLAEFYHPGKEYNVRVASRYFKGPELLVDLQDY
Z.mays PTLTDYDIRYYIYELLKALDYCHSQGIMHRDVKPHNVIMIDHELRLRLIDWGLAEFYHPGKEYNVRVASRYFKGPELLVDLQDY
A.thaliana PTLTDYDIRYYIYELLKALDFCHSQGLMHRDVKPHNVIMIDHELRLRLIDWGLAEFYHPGKEYNVRVASRYFKGPELLVDLQDY

Lpck2a-1 DYSLDMWSLGCMFAGMI FRKEPFFYGHNDHQDLVKIAKVLGTDGLNAYLNKYHIELDPHLEHLVGRHSRKPWSKF INADNQHLY
O.sativa DYSLDMWSLGCMFAGMI FRKEPFFYGHNDHQDLVKIAKVLGTEALNAYLNKYHIELDPQLEALVGRHSRKPWSKF INADNQHLY
Lpck2a-2 DYSLDMWSLGCMFAGMI FRKEPFFYGHNDHQDLVKIAKVLGTDGLNAYLNKYRIELDPQLEALVGRHSRKPWSKF INADNQHLY
T.aestivum DYSLDMWSLGCMFAGMI FRKEPFFYGHNDHQDLVKIAKVLGTDGLNAYLNKYRIELDPQLEALVGRHSRKPWSKF INADNQHLY
O.sativa DYSLDMWSLGCMFAGMI FRKEPFFYGHNDHQDLVKIAKVLGTDGLNAYLNKYRIELDPQLEALVGRHSRKPWSKF INADNQHLY
Z.mays DYSLDMWSLGCMFAGMI FRKEPFFYGHNDHQDLVKIAKVLGTDGLNAYLNKYRIELDPQLEALVGRHSRKPWSKF INADNQHLY
A.thaliana DYSLDMWSLGCMFAGL FRKEPFFYGHNDQDLVKIAKVGVDDELNAYLNKYQLDELDPQLEALVGRHSRKPWSKF INADNQHLY

Lpck2a-1 SPEAIDFLDKLLRYDHDQRLTAREAMAHYPYFLOVQAAENSRSPQ* (AB213316)
O.sativa SPEAIVDFLDKLLRYDHDQRLTAREAMAHYPYFLOVRAAENSRARPO* (AB036788.1:OsCK2a)
Lpck2a-2 SPEAIDFLDKLLRYDHDQRLTAREAMAHYPYFLOVRAAENSRTRAO* (AB213317)
T.aestivum SPEAIDFLDKLLRYDHDQRLTAREAMAHYPYFLOVRAAENSRTRAO* (AB052133: tck2a)
O.sativa SPEAIDFLDKLLRYDHDQRLTAREAMAHYPYFLOVRAAENSRPRAQ* (NM194133)
Z.mays SPEAIDFLDKLLRYDHDQRLTAREAMAHYPYFLOVRAVENSRTRPO* (Y11526.1)
A.thaliana SPEAIVDFLDKLLRYDHDQRLTAKEAMAHAYFAQVRAAETSRRMSQ* (D102460)

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primers were designed. The 1.5 kb amplicon generated by the *Lpck2a-2*EF/R primer pair also contained an intron, but this smaller amplicon was suitable for

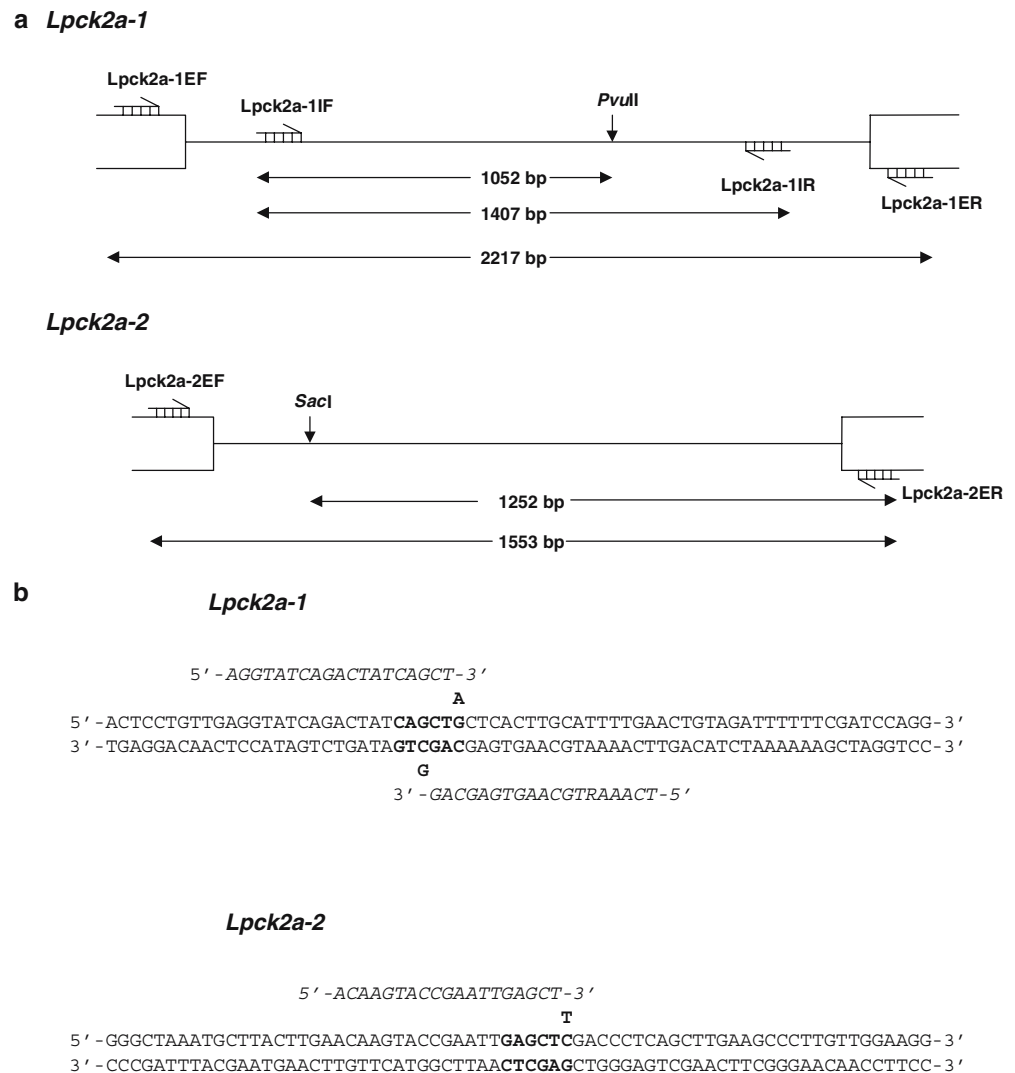
complete sequence analysis directly, from either end of the cloned sequence, when compared to the *Lpck2a-1*EF/R amplicon that was rather too large for this

purpose (Fig. 2a). The PCR products for each gene were obtained from the heterozygous parent of the p150/112 family. Following cloning, sequencing and alignment, the putative SNPs were identified. Complex haplotype structures, involving subsequently validated SNPs, suggested that the amplification products had been obtained from paralogous sequences (data not shown). However, allelic variation was observed in predicted restriction enzyme target sites within the intron of each gene (Fig. 2a). The two haplotypes for the *Lpck2a-1* gene contain a canonical *PvuII* site and a variant with SNPs at the third and sixth base position. The corresponding structures for the *Lpck2a-2* gene contain a canonical *SacI* site and a variant with an SNP at the sixth base position (Fig. 2b). SNUpe primers were designed to interrogate and validated these putative SNPs. Although both the convergently orientated SNUpe primers for the *Lpck2a-1* SNPs contained mismatches at the third base position from the 3'-terminus, this did not compromise the detection of the relevant variants.

Development of CAPS assays and genetic linkage analysis of *CK2 α* genes

The location of SNPs in diagnostic restriction enzyme sites provided the basis for simple CAPS assays suitable for screening allelic variation in the absence of specialised SNP detection chemistries and equipment platforms. Amplicon-specific PCR was performed for each gene on genomic DNA from the heterozygous parent and F₁ progeny of the p150/112 family, in order to generate single products. Following digestion with the diagnostic restriction enzyme, two genotypic classes were revealed for each assay: a class with two bands resembling the heterozygous parental genotype and a class with one (homozygous) band (Figs. 3a, 4b). The sizes of the bands were, in each case, consistent with the sequence data. Due to the one-way pseudo-testcross structure of the mapping population, both markers conform to AB \times BB allelic segregation patterns. The *Lpck2a-1* CAPS assay classified the progeny set into two groups of 45 heterozygous

Fig. 2 SNP detection in *Lpck2a* genes based on amplicon cloning and sequencing, and SNP validation. **a** Location of amplification primers within the gene structure of the *Lpck2a-1* and *Lpck2a-2* genes. The exon regions are indicated as blocks and the single intron for each gene as a line. Single sites for diagnostic restriction enzymes are indicated. The predicted sizes of amplicons and derived CAPS products are shown in bp. **b** Genomic DNA sequences surrounding diagnostic restriction enzymes sites for the *Lpck2a-1* (*PvuII*) and *Lpck2a-2* (*SacI*) genes. Variant bases within the alternative haplotypes are indicated in *bold* above and below the reference sequence. The sequences of SNUpe interrogation primers are aligned with the reference sequence and indicated in *italics*. The two SNPs in the *Lpck2a-1* *PvuII* site were interrogated in convergent orientation



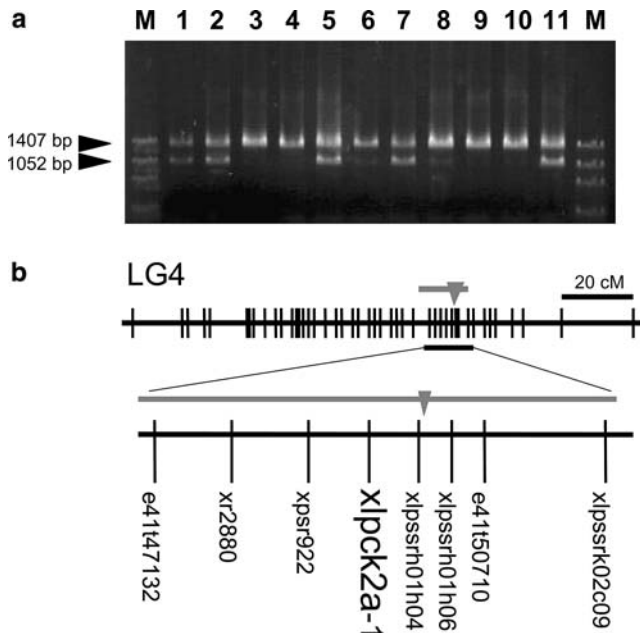


Fig. 3 Genetic mapping of the *Lpck2a-1* CAPS marker in the p150/112 reference mapping population. **a** Electrophoretic separation of segregating CAPS alleles. Lane 1 heterozygous parent and Lanes 2–11 F₁ progeny genotypes. The sizes of the A and B alleles are indicated (ca. 1.4 and 1.05 kb), with reference to the lambda DNA/*Sly*I size standard (M). **b** Interpolated location of the *xlpck2a-1* locus on LG4 of perennial ryegrass. The maximum likelihood value location of the heading date QTL from the p150/112 population (Yamada et al. 2004) is indicated by a grey arrow and the extent of one LOD unit drop from the maximum value by a grey bar. Nomenclature of genomic DNA-derived SSR (LPSSR) loci, AFLP loci and heterologous RFLP loci is as described by Jones et al. (2002a, b)

genotypes and 77 homozygous genotypes. This locus showed significant segregation distortion through deviation from 1:1 expectation at the $P < 0.05$ level. The *Lpck2a-2* CAPS assay classified the progeny into 57 heterozygous genotypes and 54 homozygous genotypes, not significantly different to the expected 1:1 segregation. The accuracy of the CAPS assays were compared to the SNUPe assays of ten randomly selected F₁ genotypes for each marker, revealing co-segregation in each case (data not shown).

The *Lpck2a-1* CAPS marker was located, with a high degree of confidence (LOD > 2.0), within the framework of the reference genetic map to the 4.3 cM interval between the markers *xlpssrh01h06* and *xpsr922* on LG4, with a most probable location adjacent to the *xlpssrh01h04* locus (Fig. 3b). The *Lpck2a-2* CAPS marker was located with a high degree of confidence to the 3.2 cM interval between *xlpssrk02e02* and *e33t50133* on LG2 (Fig. 4b). Several genomic regions on the p150/112 genetic map showed local concentrations of skewed markers. The location of the *xlpck2a-1* CAPS locus on LG4 was within such a region, consistent with its significant segregation distortion.

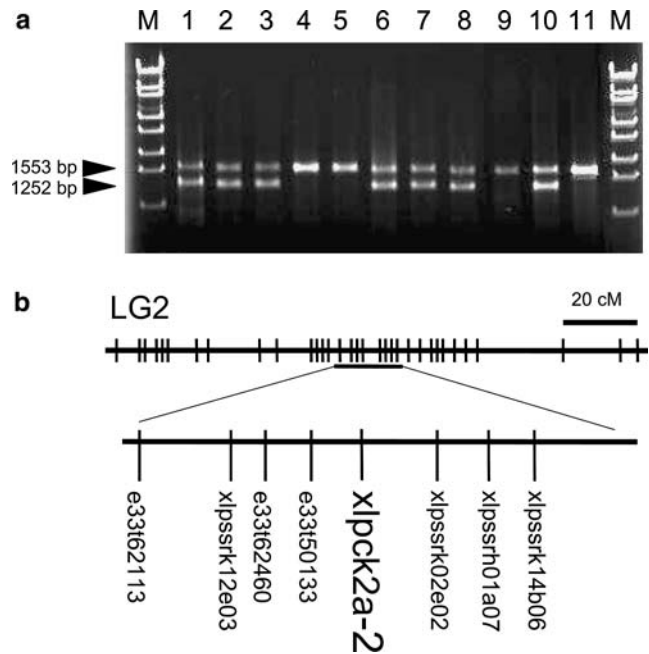


Fig. 4 Genetic mapping of the *Lpck2a-2* CAPS marker in the p150/112 reference mapping population. **a** Electrophoretic separation of segregating CAPS alleles. Lane 1 heterozygous parent and Lanes 2–11 F₁ progeny genotypes. The sizes of the A and B alleles are indicated (ca. 1.5 and 1.25 kb), with reference to the ϕ X174/*Hae*III size standard (M). **b** Interpolated location of the *xlpck2a-2* locus on LG2 of perennial ryegrass. Nomenclature of genomic DNA-derived SSR (LPSSR) loci and AFLP is as described by Jones et al. (2002a, b)

Association between allelic variation at locus-specific markers and phenotypic variation for heading date

Relationships were determined between genotypes at the two *Lpck2a* gene CAPS loci and phenotypic variation in the p150/112 population for the heading date, winter survival and electrical conductivity (as a measure of freezing tolerance) (Tables 1, 2). For the heading date, a highly significant effect ($P < 0.001$) was observed for the *xlpck2a-1* locus, such that homozygous individuals showed an earlier mean date than heterozygotes. This result is consistent with the presence of a heading date QTL, in the corresponding region of LG4 (Yamada et al. 2004). No significant effect for the heading date

Table 1 Association of *xlpck2a* CAPS loci with heading date, winter hardiness and electrical conductivity (as a measure of freezing tolerance) using analysis of variance with generalised linear modelling

Trait	<i>xlpck2a-1</i>	<i>xlpck2a-2</i>	<i>xlpck2a-1</i> × <i>xlpck2a-2</i>	R^2
Heading date	*	ns	**	0.45
Winter survival	ns	*	ns	0.33
Electrical conductivity	ns	ns	ns	–

ns not significant, $P \geq 0.05$
* $P < 0.001$; ** $P < 0.05$

Table 2 Separation of means associated with *xlpck2a* CAPS locus genotypes using Tukey's studentised range test

Trait	Locus	Homozygote class	Heterozygote class	Significance
Heading date	<i>xlpck2a-1</i>	14.5	18.2	*
	<i>xlpck2a-2</i>	15.3	16.9	ns
Winter survival	<i>xlpck2a-1</i>	1.6	1.8	ns
	<i>xlpck2a-2</i>	1.5	2.0	*
Electrical conductivity	<i>xlpck2a-1</i>	9.3	10.2	ns
	<i>xlpck2a-2</i>	9.5	9.7	ns

ns not significant, $P \geq 0.05$
* $P < 0.05$

trait was obtained for the *xlpck2a-2* locus, although a significant interaction ($P < 0.05$) between the two loci was observed. The proportion of phenotypic variation (R^2) accounted for, by variation, at the two loci was 0.45.

For the winter survival trait, a highly significant effect of allelic variation was observed for *xlpck2a-2* ($P < 0.001$), although no QTLs for this character were detected using interval mapping techniques (Yamada et al. 2004). To determine whether the less stringent test of single marker-based analysis of variance could detect genetic effects with other loci in the vicinity of *xlpck2a-2*, flanking markers were evaluated and also found to be significant at P values ranging from 0.05 to < 0.001 (data not shown). No significant effects of allelic variation at *xlpck2a-1* or between-locus interactions were observed. For electrical conductivity, no significant effect of either locus or between-locus interactions were detected.

Discussion

The completion of whole-genome sequencing programs, for model plant species such as *A. thaliana* and *O. sativa*, has permitted the identification of genes regulating various flowering control pathways (Mouradov et al. 2002; Simpson and Dean 2002). Protein phosphorylation plays an important role in the photoperiodic control of flowering that has been conserved during plant evolution. The CK2 protein kinase affects floral transition in both model species, probably through circadian clock regulation (Izawa et al. 2003; Daniel et al. 2004). The identification and genetic analysis of two perennial ryegrass CK2 α genes provides the basis for equivalent studies in an important pasture grass species. Although the sequence similarity between these genes and those from other species is high, the phylogenetic analysis of the gene products suggests that the two genes belong to different sub-families of Poaceae CK2 α proteins. The identification of a sub-cluster of maize CK2 α sequences, which is separate but close to the sub-clusters that contain each accession, may indicate the presence of other sub-families that are yet to be identified in perennial ryegrass, consistent with the genomic Southern hybridisation data.

Sequence analysis of targeted amplicons from both perennial ryegrass CK2 α genes detected SNP variation in single large introns. The amplicon cloning and sequencing strategy has been designed to eliminate

confounding effects associated with multiple heterozygosity and multiple gene origin, which are associated with direct sequencing (Forster et al. 2004), and provides direct haplotype data. Complex haplotype structures, associated with both genes, suggest that locus-specific amplification primers may have obtained sequences from several paralogous members of the CK2 α gene family. The sequence variants associated with these haplotypes may prove useful for further analysis of gene family structure.

The detection of multiple perennial ryegrass genes, along with Southern hybridisation data in this study, is consistent with the presence of small multigene families in a number of plant species such as *A. thaliana* (Mizoguchi et al. 1993) and maize (Peracchia et al. 1999; Riera et al. 2001). The two different perennial ryegrass CK2 α genes are located on separate linkage groups, based on locus-specific CAPS markers. Two CK2 α genes have also been identified from barley: one mapping in close proximity to *VrnH1* on chromosome 5H, in a region of conserved synteny with the wheat *VrnA1* gene, and the other assigned to chromosome 2H (K. Kato, personal communication). At least two rice CK2 α genes have also been identified: *OsCK2a* at the *Hd6* locus on chromosome 3 and the gene represented by the NM194133 cDNA on chromosome 7. The locations of the perennial ryegrass *Lpck2a* loci are consistent with previous comparative genetics studies. The *xlpck2a-1* CAPS locus was located to the upper region of LG4, which predominantly corresponds to the homeologous group 4 chromosomes of the Triticeae cereals. However, the upper part of LG4 contains markers that map to the wheat homeologous group-5 chromosomes (Jones 2002a). Comparisons of the genetic maps of perennial ryegrass, meadow fescue and the Triticeae, have revealed an evolutionary translocation that may have occurred before the divergence of the Poaceae grasses (Alm et al. 2003; Yamada et al. 2004; Sim et al. 2005). On this basis, it seems likely that the region close to *xlpck2a-1* will show conserved synteny with the wheat *VrnA1* region and the rice *Hd6* region on chromosome 3. A QTL for heading date variation in the p150/112 perennial population has also been observed on LG4 (Yamada et al. 2004), with a maximum log-of-odds (LOD) position close to the *xlpck2a-1* locus. Other studies have revealed the heading date and vernalisation response QTLs in this region (Armstead et al. 2004; Jensen et al. 2005).

The *xlpck2a-2* locus is located on LG2, in a region of conserved synteny with wheat 2A, 2B and 2D, barley 2H and rice chromosome 7. The latter observation is

consistent with the high level of sequence identity between *Lpck2a-2* and the rice NM194133 cDNA. However, a higher level of sequence identity with the wheat *tck2a* gene was observed for *Lpck2a-2* compared to *Lpck2a-1*. The map position of *tck2a* on wheat 5AL would be more consistent with putative orthology with *Lpck2a-1*. This apparent anomaly is probably attributable to the RFLP mapping method employed for *tck2a*, which detected major monomorphic fragments that could not be directly assigned to chromosomal locations (Kato et al. 2002). The *tck2a* cDNA sequence may, hence, correspond to loci on the wheat homoeologous group-2 chromosomes, and a paralogous sequence, more closely related to *OsCK2a*, may have been mapped to chromosome 5AL. Partial confirmatory data has been obtained from the results of comparative genomics analysis using the wheat deletion bin mapping system (Endo and Gill 1996; Qi et al. 2003). Both *Lpck2a-1* and *Lpck2a-2* identify BE498566 as the highest matching mapped wheat EST (with 88 and 94% nucleotide identity, respectively). This EST shows 99% nucleotide identity with *tck2a*, and was assigned by hybridisation analysis to a location in the interstitial bin C-2AL1-0.85 on chromosomal 2AL. Although this physical mapping data is consistent with the analysis of *Lpck2a-2*, a corresponding physical location on 5AL might be anticipated, at least for *Lpck2a-1*. This may be attributable to the current absence of a map-assigned wheat EST that is putatively orthologous to *OsCK2a* and *Lpck2a-1*.

The isolation of CK2 α genes from perennial ryegrass provides the opportunity to assess the degree of conservation of genetic mechanisms for floral induction and winter hardiness between grass and cereal species. The present study indicates that allelic variation at the *xlpcck2a-1* locus is associated with significant phenotypic variation in the heading date, similar to the observed effects of allelic variation in the *OsCK2a* gene at the *Hd6* locus in rice (Takahashi et al. 2001). The location of the maximum LOD value for the heading date on LG4 is coincident with marker *xlpsrh01h04* (Yamada et al. 2004), which co-segregates with *xlpcck2a-1*. No significant QTLs for the heading date were detected on LG2; but a re-examination of the composite interval mapping (CIM) data, in the context of the significant interaction effect between the two loci, revealed a maximum LOD value of 2.83 at 70.7 cM, close to the interpolated location of *xlpcck2a-2* (65.5–68.7 cM). As the empirically determined threshold value for this trait was 2.9 (data not shown), the QTL was not declared as significant, but the supporting evidence described in this study suggests that weak genetic effects from LG2 may contribute to flowering time variation in the p150/112 family.

The low-temperature tolerance traits were also tested for correlation with allelic variation at the *xlpcck2a* loci. The maximum LOD value for electrical conductivity was considerably more distal on LG2 than *xlpcck2a-1* and, accordingly, no association was observed. No significant QTLs for winter survival were previously detected (Yamada et al. 2004) and the

non-significant association of *xlpcck2a-1* is consistent with the maximum LOD values in this region of 1.4 and 1.5, with simple interval mapping (SIM) and CIM, respectively. The significant association of *xlpcck2a-2* with winter survival may be explained by the presence of a sub-threshold QTL for this trait on LG2, with a maximum LOD value of 2.0 at 52.6 cM. The nature of the functional sequence variation on LG2, which could account for effects on both the heading date and winter survival, is not as obvious as for LG4. However, major photoperiod response genes are located on the short arms of Triticeae group-2 chromosomes (Law et al. 1978), in regions of conserved synteny with the upper part of perennial ryegrass LG2 (Jones et al. 2002a). The barley *Ppd-H1* gene is located on 2HS, adjacent to the marker *xbcd221* (Dunford et al. 2002). Based on comparative analysis between barley and oat (*Avena sativa* L.) (O'Donoghue et al. 1992), the closest comparative marker to the putative ortholocus in perennial ryegrass would be *xcdo38.1* at 34.6 cM. Although *Lpck2a-2* is consequently unlikely to be a candidate for the control of photoperiodic sensitivity, allelic variation at such a linked gene could account for the observed effects, which are consistent with the known correlation between later flowering and enhanced freezing tolerance.

The next generation of molecular genetic markers for forage grasses will be derived from expressed sequences, with an emphasis on functionally defined genes associated with biochemical and physiological processes that are likely to be correlated with target phenotypic traits (Faville et al. 2004). SNP genotyping provides a highly efficient method for the high-throughput screening of such markers, providing the basis for the direct selection of superior allele content at target loci (Forster et al. 2004). The sequence data from allelic variants of *Lpck2a-1* and *Lpck2a-2* in the reference population provides the basis for the validation of SNP loci in other populations and large-scale genotyping of germplasm resources showing flowering time and low temperature tolerance variation.

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References

- Alm V, Fang C, Busso CS, Devos KM, Vollan K, Grieg Z, Rognli OA (2003) A linkage map of meadow fescue (*Festuca pratensis* Huds.) and comparative mapping with other Poaceae species. *Theor Appl Genet* 108:25–40

- Armstead IP, Turner LB, King IP, Cairns AJ, Humphreys MO (2002) Comparison and integration of genetic maps generated from F₂ and BC₁-type mapping populations in perennial ryegrass. *Plant Breed* 121:501–507
- Armstead IP, Turner LB, Farrell M, Skot, Gomez P, Montoya T, Donnison IS, King IP, Humphreys MO (2004) Synteny between a major heading-date QTL in perennial ryegrass (*Lolium perenne* L.) and the *Hd3* heading-date locus in rice. *Theor Appl Genet* 108:822–828
- Bert PF, Charmet G, Sourdille P, Hayward MD, Balfourier F (1999) A high-density molecular map for ryegrass (*Lolium perenne*) using AFLP markers. *Theor Appl Genet* 99:445–452
- Cattivelli L, Baldi P, Crosatti C, Di Fonzo N, Faccioli P, Grossi M, Mastrangelo AM, Pecchioni N, Stanca AM (2002) Chromosome regions and stress-related sequences involved in resistance to abiotic stress in Triticeae. *Plant Mol Biol* 48:649–665
- Cogan NOI, Smith KF, Yamada T, Francki MG, Vecchies AC, Jones ES, Spangenberg GC, Forster JW (2005) QTL analysis and comparative genomics of herbage quality traits in perennial ryegrass (*Lolium perenne* L.). *Theor Appl Genet* 110:364–380
- Daniel X, Gugano S, Tobin EM (2004) CK2 phosphorylation of CCA1 is necessary for its circadian oscillator function in *Arabidopsis*. *Proc Natl Acad Sci USA* 101:3292–3297
- Dubcovsky J, Lijavetzky D, Appendino L, Tranquilli G (1998) Comparative RFLP mapping of *Triticum monococcum* genes controlling vernalization requirement. *Theor Appl Genet* 97:968–975
- Dumsday JL, Smith KF, Forster JW, Jones ES (2003) SSR-based genetic linkage analysis of resistance to crown rust (*Puccinia coronata* Corda f. sp. *lolii*) in perennial ryegrass (*Lolium perenne* L.). *Plant Pathol* 52:628–637
- Dunford RP, Yano M, Kurata N, Sasaki T, Huestis G, Rocheford T, Laurie D (2002) Comparative mapping of the barley *Ppd-H1* photoperiod response gene region, which lies close to a junction between two rice linkage segments. *Genetics* 161:825–834
- Endo TR, Gill BS (1996) The deletion stocks of common wheat. *J Hered* 87:295–307
- Faville M, Vecchies AC, Schreiber M, Drayton MC, Hughes LJ, Jones ES, Guthridge KM, Smith KF, Sawbridge T, Spangenberg GC, Bryan GT, Forster JW (2004) Functionally-associated molecular marker map construction in perennial ryegrass (*Lolium perenne* L.). *Theor Appl Genet* 110:12–32
- Francia E, Rizza F, Cattivelli L, Stanca AM, Galiba G, Toth B, Hayes PM, Skinner JS, Pecchioni N (2004) Two loci on chromosome 5H determine low-temperature tolerance in a 'Nure' (winter) × 'Tremois' (spring) barley map. *Theor Appl Genet* 108:670–680
- Forster JW, Jones ES, Kölliker R, Drayton MC, Dumsday J, Dupal MP, Guthridge KM, Mahoney NL, van Zijl de Jong E, Smith KF (2001) Development and implementation of molecular markers for forage crop improvement. In: Spangenberg G (ed) *Molecular breeding of forage crops*. Kluwer Academic Press, Dordrecht, pp 101–133
- Forster JW, Jones ES, Batley J, Smith KF (2004) Molecular marker-based genetic analysis of pasture and turf grasses. In: Hopkins A, Wang Z-Y, Mian R, Sledge M, Barker RE (eds) *Molecular breeding of forage and turf*. Kluwer Academic Press, Dordrecht, pp 197–238
- Humphreys MO, Eagles CF (1988) Assessment of perennial ryegrass (*Lolium perenne* L.) for breeding. I. Freezing tolerance. *Euphytica* 38:75–84
- Izawa T, Takahashi Y, Yano M (2003) Comparative biology comes into bloom: genomic and genetic comparison of flowering pathways in rice and *Arabidopsis*. *Curr Opin Plant Biol* 6:113–120
- Jensen LB, Andersen JR, Frei U, Xing Y, Taylor C, Holm PB, Lübberstedt T (2005) QTL mapping of vernalization response in perennial ryegrass (*Lolium perenne* L.) reveals co-location with an orthologue of wheat *VRN1*. *Theor Appl Genet* 110:527–536
- Jones ES, Mahoney NL, Hayward MD, Armstead IP, Jones JG, Humphreys MO, King IP, Kishida T, Yamada T, Balfourier F, Charmet C, Forster JW (2002a) An enhanced molecular marker-based map of perennial ryegrass (*Lolium perenne* L.) reveals comparative relationships with other Poaceae species. *Genome* 45:282–295
- Jones ES, Dupal MD, Dumsday JL, Hughes LJ, Forster JW (2002b) An SSR-based genetic linkage map for perennial ryegrass (*Lolium perenne* L.). *Theor Appl Genet* 105:577–584
- Kato K, Miura H, Sawada S (1999) Comparative mapping of the wheat *Vrn-A1* region with the rice *Hd-6* region. *Genome* 42:204–209
- Kato K, Kidou S, Miura H, Sawada S (2002) Molecular cloning of the wheat *CK2α* gene and detection of its linkage with *Vrn-A1* on chromosome 5A. *Theor Appl Genet* 104:1071–1077
- Lander ES, Green P, Abrahamson J, Barlow A, Daly M J, Lincoln SE, Newburg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. *Genomics* 1:174–181
- Law CN, Sutka J, Worland AJ (1978) A genetic study of day-length response in wheat. *Heredity* 41:185–191
- Lee Y, Lloyd AM, Roux SJ (1999) Antisense expression of the CK2 α -subunit gene in *Arabidopsis*. Effects on light-regulated gene expression and plant growth. *Plant Physiol* 119:989–1000
- Limin AE, Fowler DB (2002) Developmental traits affecting low-temperature tolerance response in near-isogenic lines for the vernalization locus *Vrn-A1* in wheat (*Triticum aestivum* L. em Thell.). *Ann Bot* 89:579–585
- Litchfield DW, Lüscher B (1993) Casein kinase II in signal transduction and cell cycle regulation. *Mol Cell Biochem* 127/128:187–199
- Meggio F, Pinna LA (2003) One-thousand-and-one substrates of protein kinase CK2? *FASEB J* 17:349–368
- Mizoguchi T, Yamaguchi-Shinozaki K, Hayashida N, Kamada H, Shinozaki K (1993) Cloning and characterization of two cDNAs encoding casein kinase II catalytic subunits in *Arabidopsis thaliana*. *Plant Mol Biol* 21:279–289
- Mouradov A, Cremer F, Coupland G (2002) Control of flowering time: interacting pathways as a basis for diversity. *Plant Cell* 14(Suppl):S111–S130
- Murray MG, Thompson WF (1980) The isolation of high molecular weight plant DNA. *Nucleic Acids Res* 8:4321–4325
- O'Donoghue LS, Wang Z, Roeder M, Kneen B, Leggett M, Sorrells ME, Tanksley SD (1992) An RFLP-based linkage map of oats based on a cross between two diploid taxa (*Avena atlantica* × *A. hirtula*). *Genome* 35:765–771
- Pan A, Hayes PM, Chen F, Chen THH, Blake T, Wright S, Karsai I, Bedö Z (1994) Genetic analysis of the components of winter-hardiness in barley (*Hordeum vulgare* L.). *Theor Appl Genet* 89:900–910
- Peracchia G, Jensen AB, Culiàñez-Macià FA, Grosset J, Goday A, Issinger O-G, Pagès M (1999) Characterization, subcellular localization and nuclear targeting of casein kinase 2 from *Zea mays*. *Plant Mol Biol* 40:199–211
- Pinna LA (1990) Casein kinase 2: an 'eminence grise' in cellular regulation? *Biochim Biophys Acta* 1054:267–284
- Qi L, Echalié B, Friebe B, Gill BS (2003) Molecular characterization of a set of wheat deletion stocks for use in chromosome bin mapping of ESTs. *Funct Integr Genomics* 3:39–55
- Riera M, Peracchia G, Nadal E de, Ariño J, Pagès (2001) Maize protein kinase CK2: regulation and functionality of three β regulatory subunits. *Plant J* 25:365–374
- Sim S, Chang T, Curley J, Warnke SE, Barker R, Jung G (2005) Chromosomal rearrangements differentiating the ryegrass genome from the Triticeae, oat and rice genomes using common heterologous RFLP probes. *Theor Appl Genet* 110:1011–1019
- Simpson GG, Dean C (2002) *Arabidopsis*, the Rosetta stone of flowering time? *Science* 296:285–289
- Takahashi Y, Shomura A, Sasaki T, Yano M (2001) *Hd6*, a rice quantitative trait locus involved in photoperiod sensitivity, encodes the α subunit of protein kinase CK2. *Proc Natl Acad Sci USA* 98:7922–7927
- Tuazon PT, Traugh JA (1991) Casein kinases I and II—multipotential serine protein kinases: structure, function, and regulation. *Adv Sec Mess Phos Res* 23:123–164

- Wilkins PW, Humphreys MO (2003) Progress in breeding perennial forage grasses for temperate agriculture. *J Agr Sci* 140:129–150
- Yamada T, Jones ES, Nomura T, Hisano H, Shimamoto Y, Smith KF, Hayward MD, Forster JW (2004) QTL analysis of morphological, developmental and winter hardiness-associated traits in perennial ryegrass (*Lolium perenne* L.). *Crop Sci* 44:925–935
- Yamamoto T, Lin H, Sasaki T, Yano M (2000) Identification of heading date quantitative locus *Hd6* characterization of its epistatic interaction with *Hd2* in rice using advanced back-cross progeny. *Genetics* 154:885–891