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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 flowering time. Two putative CK2α 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 design 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 previous 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 further study of the role of CK2α 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*

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). 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 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 CK2α 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 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 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 COR (COld-Regulated) and CBF (C-Repeat Binding Factor) gene families (Francia et al. 2004). Putative orthologous sequences are observed on the long arms of the group 5 homoeologous 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 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 CK2α 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 time to 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 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 F_2 populations (Jones et al. 2002a; Jones et al. 2002b; 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 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 CK2α 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 CK2α 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 Poeae 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 CK2α cDNA clones

Perennial ryegrass (cultivar [cv.] Aberystwyth S23) plants were grown in a controlled climate chamber (16 hours day length, 22°C/18°C day/night) for 30 days and were cold-acclimated (8 hours 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 ZAP Express® cDNA Synthesis Kit and ZAP Express® cDNA Gigapack® III Gold Cloning Kit (Stratagene) according to manufacturer's instructions. A CK2α gene–specific probe was obtained by PCR amplification from this cDNA library using the primer pair combination tck2aF (5'-ATCATGAGCTTCGAAAACTC-3') and tck2aR (5'-TCACGTGCGGTGAGCCTATC-3') designed on the basis of the DNA sequence of the wheat CK2α 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 c. 3×10^4 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

XLOLR strain. The nucleotide sequences of the inserts were determined using the CEQ^{TM} 8000 Genetic Analysis System (Beckman Coulter).

Molecular phylogenetics analysis

CK2 α-subunit protein sequences were obtained using BLAST applications in 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 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 (DraI, EcoRI, HindIII, SacI and XbaI). Digested DNA was transferred to Hybond N or N$^+$ nylon membranes (GE Healthcare) prior to Southern hybridisation using the perennial ryegrass CK2α 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, Jones et al. 2002b; 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

Primer pairs were designed to amplify genomic regions specific to each CK2α gene. For the Lpck2a-1 gene, initial primer design was directed to exonic sequences (Lpck2a-1EF: 5’-CAGCCAGCTTCGTATTTTCG-3’; Lpck2a-1ER: 5’-CTGGGAGTCTTCGAATGTTG-3’), and subsequent nested design was directed to intron sequences (Lpck2a-1IF: 5’-TGATCACTGTCAATTGCAGC-3’; Lpck2a-1IR: 5’-ACAATGGAAGGTCTCCATCA-3’). For the Lpck2a-2 gene, the primers designed from exon sequences were designated Lpck2a-2EF (5’-ATAGACTGGGGCCTTGCTGA-3’) and Lpck2a-2ER (5’-GGTGATCATAGCGCAGAAGC-3’). The conditions for locus-specific PCR amplification were: 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.25 nM each primer, and 0.25 units Hotstar polymerase (Qiagen) per 20 μl reaction. Cycling conditions consisted of an initial polymerase activation step at 94°C for 15 minutes, 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 manufacturer's instructions. Transformants were selected on LB medium containing carbenicillin as the selective antibiotic, and recombinants were identified by colony PCR as described above. Plasmid template was prepared for DNA sequencing using the TempliPhi™ procedure (GE Healthcare) according to manufacturer's instructions. DNA sequencing was performed using DYEnamic ET dye terminator cycle sequencing reagents (GE Healthcare) with 2 μl of amplified product and 500 nM T3 or T7 universal primers in a 10 μl reaction. Capillary electrophoresis was performed using MegaBACE 1000 or 4000 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 Heathcare genotyping kit according to manufacturer's instructions.

SNP genotyping was performed using the MegaBACE 1000 capillary electrophoresis platform (GE Healthcare) with the following run parameters: injection at 12 kV for 20 sec, with an interval time of 90 sec 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). PCR amplicons generated using the Lpck2a-1IF/R primer pair were digested with the restriction enzyme PvuII, while PCR amplicons generated using the Lpck2a-2EF/R primer pair were digested with the restriction enzyme SacI. The digested fragments were separated in 1% (w/v) horizontal agarose gels using 1 x Tris-Borate-EDTA (TBE) buffer. DNA fragments were visualized and photographed under UV light following staining with 0.5 μg/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 xlpck2a loci were interpolated into 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

Comparative genomics analysis of perennial ryegrass CK2α 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 CK2α 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 CK2α 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%) identity with a second rice CK2α gene variant (represented by GenBank Accession No. NM94133), although sequence identity in comparison to OsCK2a was still high (88%).

Figure 1

Alignment of the deduced amino acid sequences of the perennial ryegrass CK2α genes and related sequences from public databases was performed (Figure 1A). The Lpck2a-1 and Lpck2a-2 primary protein sequences showed 91.0% and 92.5% identity, respectively, with an A. thaliana CK2α 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 CK2α genes. Phylogenetic relationships between multiple CK2α protein sequences were determined (Figure 1B). Essentially similar relationships were obtained through 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 only sequences from maize (Zea mays L.).

Southern hybridisation analysis using both CK2α 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 CK2α genes, containing at least two sub-families.

SNP detection and validation

Figure 2

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 primers were designed. The 1.5 kb amplicon generated by the Lpck2a-2EF/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, compared to the Lpck2a-1EF/R amplicon which was rather too large for this purpose (Figure 2A). PCR products for each gene were obtained from the heterozygous parent of the p150/112 family. Following cloning, sequencing and alignment, putative SNPs were identified. Complex haplotype structures involving subsequently validated SNPs suggested that 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 (Figure 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 a SNP at the sixth base position (Figure 2B). SNuPe primers were designed to interrogate and validated these putative SNPs. Although the convergently orientated SNuPe primers for the Lpck2a-1 SNPs both 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 CK2α genes

Figure 3

Figure 4

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 F_1 progeny of the p150/112 family, 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 (Figures 3A and 4A). The sizes of the bands were in each case consistent with sequence data. Because of the one-way pseudo-testcross structure of the mapping population, both markers conform to AB x BB allelic segregation patterns. The Lpck2a-1 CAPS assay classified the progeny set into two groups of 45 heterozygous 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 SNuPe assays of 10 randomly selected F_1 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 (Figure 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 (Figure 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.

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

Table 1

Table 2

Relationships were determined between genotypes at the two Lpck2a gene CAPS loci and phenotypic variation in the p150/112 population for heading date, winter survival and electrical conductivity (as a measure of freezing tolerance) (Table 1 and Table 2). For 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 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 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). Identification and genetic analysis of two perennial ryegrass CK2α 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, phylogenetic analysis of the gene products suggests that the two genes belong to different sub-families of Poaceae CK2α proteins. The identification of a sub-cluster of maize CK2α sequences that 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 CK2α 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 that 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 CK2α 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 CK2α genes are located on separate linkage groups, based on locus-specific CAPS markers. Two CK2α 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, the other assigned to chromosome 2H (K. Kato, pers. comm.). At least two rice CK2α 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 which may have occurred before the divergence of the Poeae 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 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 CK2α 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 xlpck2a-1 locus is associated with significant phenotypic variation in 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 maximum LOD value for heading date on LG4 is coincident with marker xlpssrh01h04 (Yamada et al. 2004), which co-segregates with xlpck2a-1. No significant QTLs for heading date were detected on LG2, but re-examination of 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 xlpck2a-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 xlpck2a loci. The maximum LOD value for electrical conductivity was considerably more distal on LG2 than xlpck2a-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 xlpck2a-1 is consistent with maximum LOD values in this region of 1.4 and 1.5 with simple interval mapping (SIM) and CIM, respectively. The significant association of xlpck2a-

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 functional sequence variation on LG2 that could account for effects on both 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 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 validation of SNP loci in other populations, and large-scale genotyping of germplasm resources showing flowering time and low temperature tolerance variation.

Acknowledgments

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Figure Legends

Figure 1

A. Alignment of deduced amino acid sequences for CK2α genes. The serine-threonine protein kinase site (grey shade) and the ATP-binding site (box) are indicated. Asterices 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 CK2α-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).

Figure 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.

Figure 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; Lanes 2-11: F_1 progeny genotypes. The sizes of the A and B alleles are indicated (c. 1.4 and 1.05 kb), with reference to the lambda DNA/StyI 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 1 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).

Figure 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; Lanes 2-11: F_1 progeny genotypes. The sizes of the A and B alleles are indicated (c. 1.5 and 1.25 kb), with reference to the φX174/HaeIII 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).

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	xlpck2a-1	xlpck2a-2	xlpck2a-1 x xpck2a-2	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	xlpck2a-1	14.5	18.2	*
	xlpck2a-2	15.3	16.9	ns
Winter survival	xlpck2a-1	1.6	1.8	ns
	xlpck2a-2	1.5	2.0	*
Electrical conductivity	xlpck2a-1	9.3	10.2	ns
	xlpck2a-2	9.5	9.7	ns

ns = not significant, $P \geq 0.05$

* = $P < 0.05$

Figure 1A

```
Lpck2a-1    MSAAPPSKRPPASFVFSTAAAVLIAALASSLIALSPRQAPPAAALRPIMSKARVYTDVNVVRPKEYWDYEALTVQWGEQDDYEV
O.sativa                                                    MSKARVYADVNVLRPKEYWDYEALTVQWGEQDDYEV
Lpck2a-2                                                    MSKAKVYADVNVVRPKEYWDYEALAVQWGEQDDYEV
T.aestivum                                                  MSKARVYADVNVVRPKEYWDYEALAVQWGEQDDYEV
O.sativa                                                    MSKARVYTDVNVLRPKEYWDYEALTVQWGEQDDYEV
Z.mays                                                      MSKAKVYTDVNVLRPKEYWDYEALTVQWGEQDDYEV
A.thaliana                                                  MSKARVYTEVNVIRPKDYWDYESLIVQWGEQDDYEV

Lpck2a-1    VRKVGRGKYSEVFEGINVTNSEKCVIKILKPVKKKKIKREIKILQNICGGPNIIKLLDIVRDQHSKTPSLIFEYVNNTDFKVLY
O.sativa    VRKVGRGKYSEVFEGINVNNNEKCIIKILKPVKKKKIKREIKILQNLCGGPNIVKLLDIVRDQHSKTPSLIFEYVNNTDFKVLY
Lpck2a-2    VRKVGRGKYSEVFEGINVNNNEKCVIKILKPVKKKKIKREIKILQNLCGGPNIVKLLDIVRDQHSKTPSLIFEYINNTDFKVLY
T.aestivum  VRKVGRGKYSEVFEGINVNNNEKCVIKILKPVKKKKIKREIKILQNLCGGPNIVKLLDIVRDQHSKTPSLIFEYINNTDFKVLY
O.sativa    VRKVGRGKYSEVFEGINVNNNEKCIIKILKPVKKKKIKREIKILQNLCGGPNIVKLLDIVRDQHSKTPSLIFEYVNNTDFKVLY
Z.mays      VRKVGRGKYSEVFEGINVNNNEKCIIKILKPVKKKKIKREIKILQNLCGGPNIVKLLDIVRDQHSKTPSLIFEFVNNTDFKVLY
A.thaliana  VRKVGRGKYSEVFEGINVNSKEKCIIKILKPVKKKKIRREIKILQNLCGGPNIVKLLDVVRDQHSKTPSLIFEYVNSTDFKVLY

Lpck2a-1    PTLTDYDIRYYLYELLKALDYCHSQGIMHRDVKPHNVMIDHELRKLRLIDWGLAEFYHPGKEYNVRVASRYFKGPELLVDLQDY
O.sativa    PTLTDYDIRYYIYELLKALDYCHSQGIMHRDVKPHNVMIDHELRKLRLIDWGLAEFYHPGKEYNVRVASRYFKGPELLVDLQDY
Lpck2a-2    PTLTDYDIRYYIYELLKALDYCHSQGIMHRDVKPHNVMIDHELRKLRLIDWGLAEFYHPGKEYNVRVASRYFKGPELLVDLQDY
T.aestivum  PTLTDYDIRYYIYELLKALDYCHSQGIMHRDVKPHNVMIDHELRKLRLIDWGLAEFYHPGKEYNVRVASRYFKGPELLVDLQDY
O.sativa    PTLTDYDIRYYIYELLKALDYCHSQGIMHRDVKPHNVMIDHELRKLRLIDWGLAEFYHPGKEYNVRVASRYFKGPELLVDLQDY
Z.mays      PTLTDYDIRYYIYELLKALDYCHSQGIMHRDVKPHNVMIDHELRKLRLIDWGLAEFYHPGKEYNVRVASRYFKGPELLVDLQDY
A.thaliana  PTLTDYDIRYYIYELLKALDFCHSQGLMHRDVKPHNVMIDHELRKLRLIDWGLAEFYHPGKEYNVRVVSRYFKGPELLVDLQDY

Lpck2a-1    DYSLDMWSLGCMFAGMIFRKEPFFYGHDNHDQLVKIAKVLGTDGLNAYLKKYHIELDPHLEHLVGRHSRKPWSKFINADNQHLV
O.sativa    DYSLDMWSLGCMFAGMIFRKEPFFYGHDNHDQLVKIAKVLGTEALNAYLNKYHIELDPQLEALVGRHSRKPWSKFINADNQHLV
Lpck2a-2    DYSLDMWSLGCMFAGMIFRKEPFFYGHDNHDQLVKIAKVLGTDGLNAYLNKYRIELDPQLEALVGRHSRKPWSKFINADNQHLV
T.aestivum  DYSLDMWSLGCMFAGMIFRKEPFFYGHDNHDQLVKIEKVLGTDGLNVYLNKYRIELDPQLEALVGRHSRKPWSKFINADNQHLV
O.sativa    DYSLDMWSLGCMFAGMIFRKEPFFYGHDNHDQLVKIAKVLGTDSLNSYLNKYRIELDPQLEALVGRHSRKPWSKFINADNQHLV
Z.mays      DYSLDMWSLGCMFAGMIFRKEPFFYGHDNHDQLVKIAKVLGTDGLNAYLNKYHIELDPQLEALVGRHSRKPWSKFMNADNQHLV
A.thaliana  DYSLDMWSLGCMFAGLLFRKEPFFYGHDNQDQLVKIAKGVGTDELNAYLNKYQLELDPQLEALVGRHSRKPWSKFINADNQHLI

Lpck2a-1    SPEAIDFLDKLLRYDHQDRLTAREAMAHPYFLQVKAAENSRSRPQ*   (AB213316)
O.sativa    SPEAVDFLDKLLRYDHQDRLTAREAMAHPYFLQVRAAENSRARPQ*   (AB036788.1:OsCK2a )
Lpck2a-2    SPEAIDFLDKLLRYDHQDRLTAREAMAHPYFLQVRAAENSRTRAQ*   (AB213317)
T.aestivum  SPEAIDFLDKLLRYDHQDRLTAREAMAHPYFLQVRAAENSRTRAQ*   (AB052133: tck2a)
O.sativa    SPEAIDFLDKLLRYDHQDRLTAREAMAHPYFLQVRAAENSRPRAQ*   (NM194133 )
Z.mays      SPEAIDFLDKLLRYDHQDRLTAREAMAHPYFLQVRAVENSRTRPQ*   (Y11526.1)
A.thaliana  SPEAVDFLDKLLRYDHQDRLTAKEAMAHAYFAQVRAAETSRMRSQ*   (D102460)
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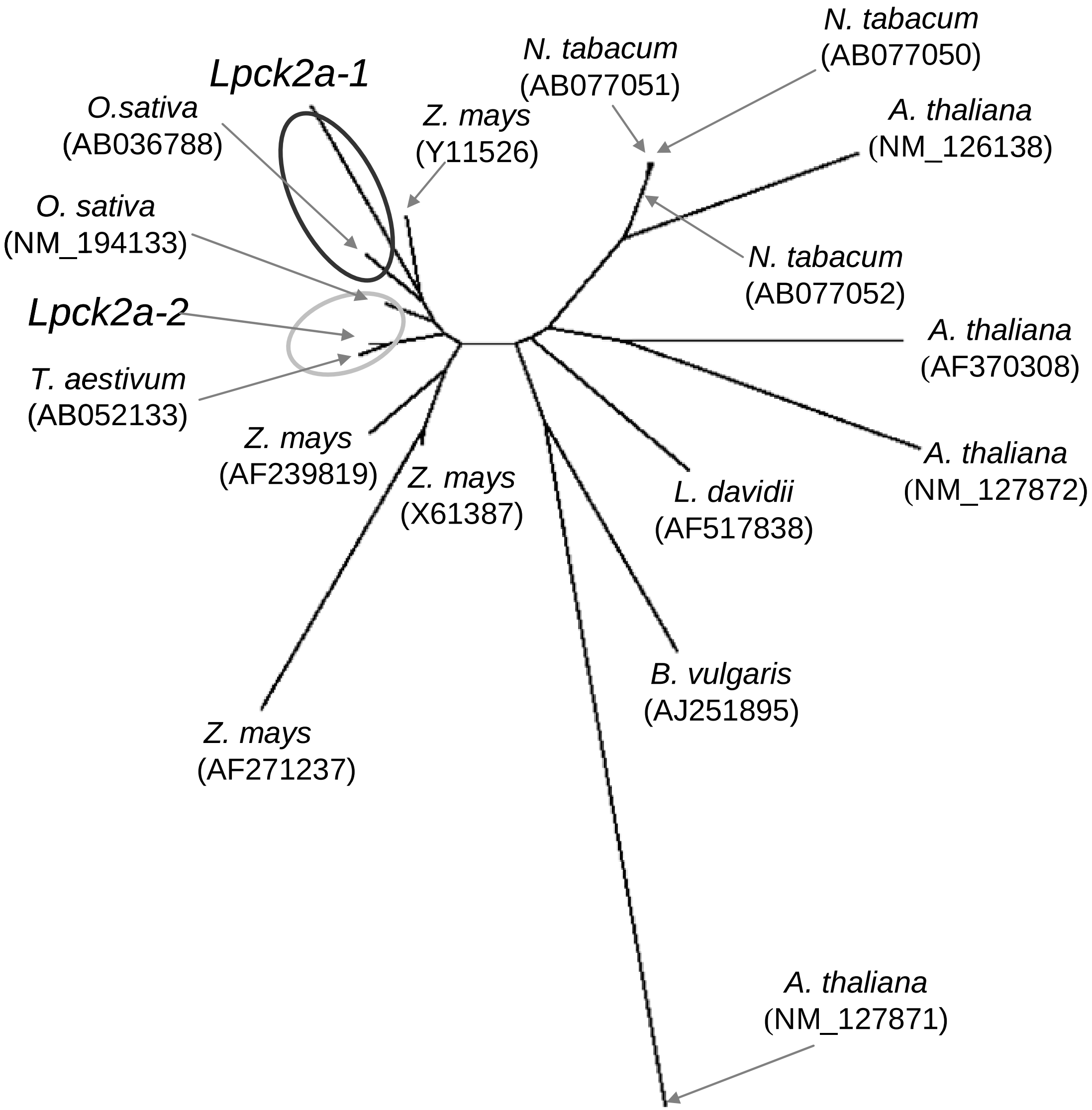
Figure 1B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Z. mays
(AF239819)
Z. mays
(X61387)
Z. mays
(AF271237)
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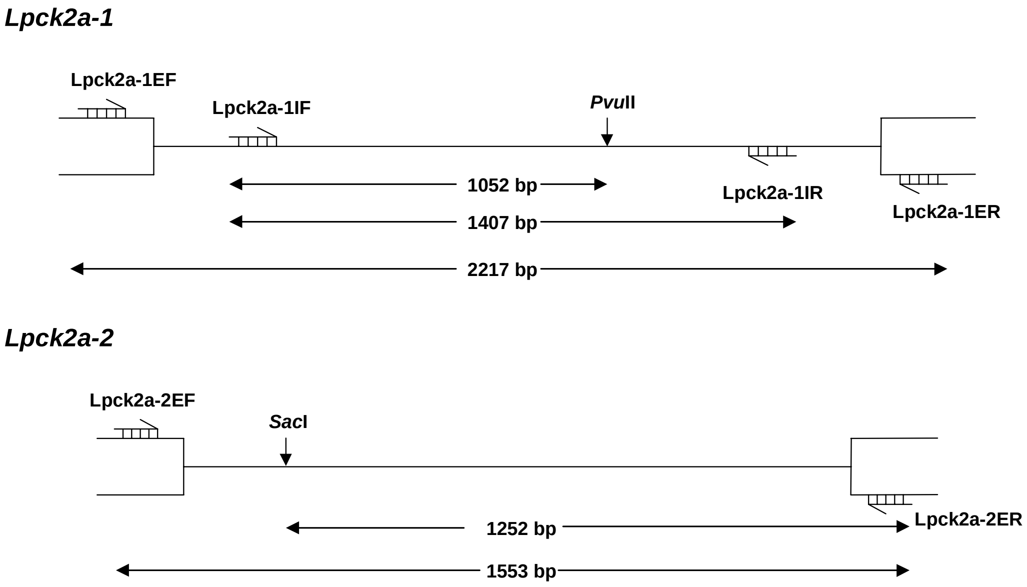
Figure 2

A)



B)

Lpck2a-1

5′-*AGGTATCAGACTATCAGCT-3′*
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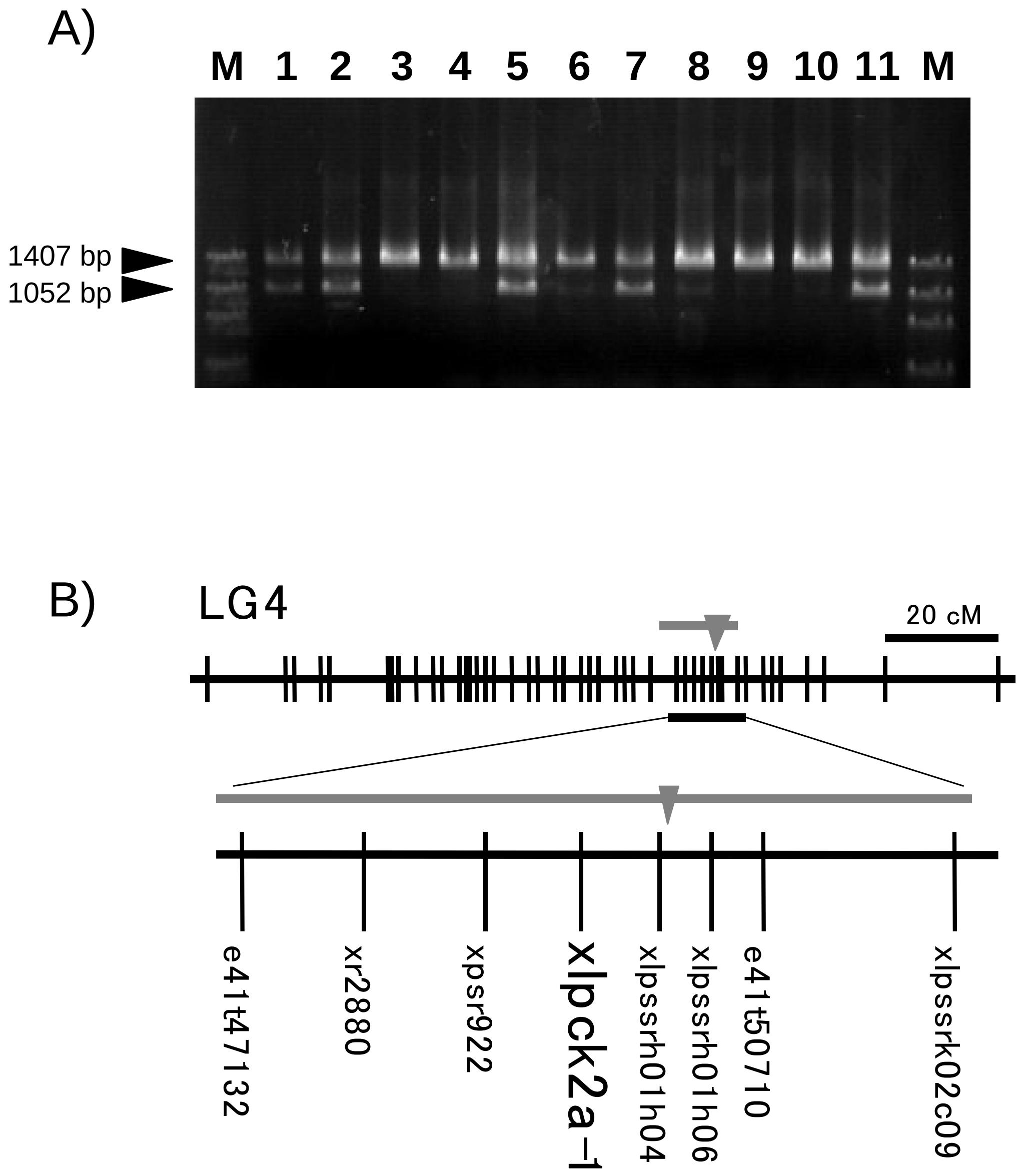
3′-TGAGGACAACTCCATAGTCTGATA**GTCGAC**GAGTGAACGTAAAACTTGACATCTAAAAAAGCTAGGTCC-3′
G
3′-*GACGAGTGAACGTRAAACT-5′*

Lpck2a-2

5′-ACAAGTACCGAATTGAGCT-3′
T
5′-GGGCTAAATGCTTACTTGAACAAGTACCGAATT**GAGCTC**GACCCTCAGCTTGAAGCCCTTGTTGGAAGG-3′
3′-CCCGATTTACGAATGAACTTGTTCATGGCTTAA**CTCGAG**CTGGGAGTCGAACTTCGGGAACAACCTTCC-3′

Figure 3



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4
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6
7
8
9
10
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M
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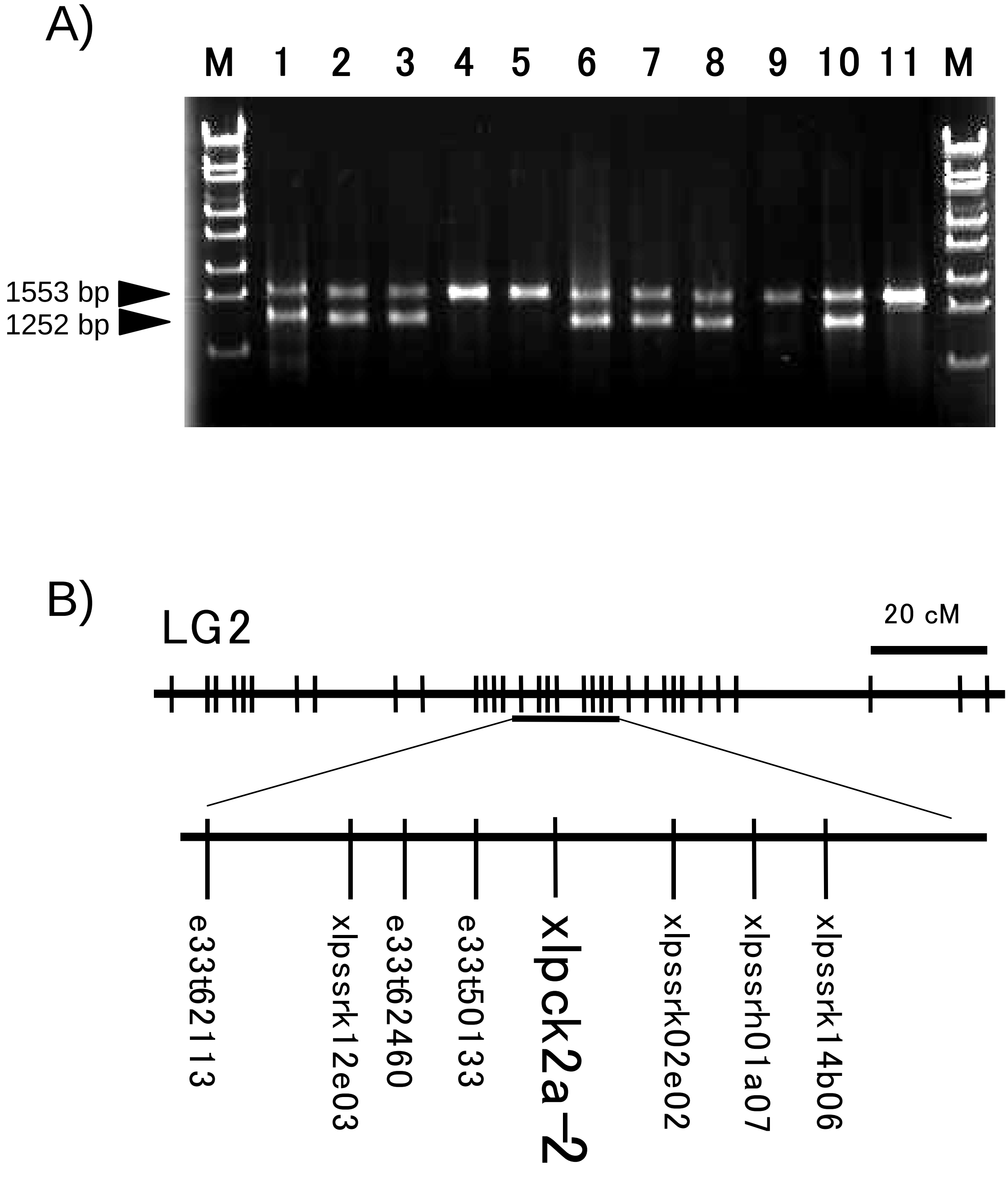
Figure 4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