Reference Transcriptome Assembly and Annotation for Perennial Ryegrass

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1 **NOTE**

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Abstract

RNA-Seq methodology has been used to generate a comprehensive transcriptome sequence resource for perennial ryegrass, an important temperate pasture grass species. A total of 931,547,255 reads were obtained from libraries corresponding to 19 distinct tissue samples, including both vegetative and reproductive stages of development. Assembly of data generated a final filtered reference set of 48,713 contigs and scaffolds. The transcriptome resource will support whole genome sequence assembly, comparative genomics, implementation of genotyping-by-sequencing (GBS) methods based on transcript sampling and identification of candidate genes for multiple biological functions.

Keywords: *Lolium*, pasture, RNA-Seq, unigene, candidate gene, breeding

1 Introduction

2 Perennial ryegrass (*Lolium perenne* L.) is a globally important pasture grass of
 3 temperate livestock production systems. Perennial ryegrass is a member of the
 4 Pooideae (cool-season) sub-family of the grass and cereal family Poaceae (Soreng
 5 et al. 2015), with a chromosomal constitution of $2n = 2x = 14$ and an estimated
 6 haploid genome size of c. 2.6 Gbp (Kopecký et al. 2010). A number of genomic
 7 resources have previously been generated for this species, to support applications
 8 such as transgenic modification (Tu et al. 2010) and genomics-assisted breeding
 9 (Forster et al. 2008). Several comprehensive expressed sequence tag (EST)
 10 collections (Sawbridge et al. 2003; Asp et al. 2007) were obtained by Sanger
 11 sequencing. More recently, RNA-Seq methodology has been used for *de novo*
 12 transcriptome assembly in perennial ryegrass (Farrell et al. 2014) and the closely
 13 related species, Italian ryegrass (*Lolium multiflorum* Lam.) (Stočes et al. 2016).
 14 Given that a draft genome assembly for perennial ryegrass has also been generated
 15 (Byrne et al. 2015), but genome coverage is incomplete, further efforts to define a
 16 comprehensive unigene set are desirable, and sampling of the gene-space by
 17 transcriptome offers an efficient and cost-effective solution. This study describes the
 18 generation, characterisation and properties of RNA-Seq data from multiple tissue
 19 types of single perennial ryegrass genotype, as a resource for use in applied pasture
 20 plant improvement.

21

22 Materials and methods

23 A total of 19 distinct tissue samples (Table 1) were obtained from a single plant
 24 genotype derived from the cultivar Grasslands Impact (AgResearch, New Zealand),
 25 designated Impact₀₄ (Kaur et al. 2015). RNA extraction for all samples was

1 performed using the RNeasy plant kit following manufacturer's instructions (Qiagen,
2 Hilden, Germany) followed by the TruSeq RNA library prep kit following
3 manufacturer's instructions (Illumina Inc., San Diego, California, USA). Libraries
4 were evaluated using the Bioanalyser (Agilent Technologies) and pooled and
5 quantified using the KAPA library quantification kit (KAPA Biosystems, Cape Town,
6 South Africa). Sequencing data was generated using the Illumina HiSeq platform
7 (Illumina Inc., San Diego, California, USA). Data was assembled through use of
8 SOAPdenovoTranscriptome v1.05 (Xie et al., 2014). Scaffolds that were identified as
9 forks, bubbles or complex were individually passed into the CAP3 assembly program
10 (Huang and Madan, 1999) in order to resolve loci into single contigs.

11

12 **Results**

13 A total of 931,547,255 reads were used for sequence assembly. A range of k-mers
14 were empirically evaluated, and a final value of 51 was found to be optimal using a
15 total of 92.3% of reads in the assembly. A total of 480,391 contigs and scaffolds
16 were generated. Filtering of this initial assembly on the basis of sequence length
17 removed 156,810 entries <149 bp in length, taken as a minimum of the basis of
18 output characteristics, such that shorter contigs represented a single, or potentially
19 partial, sequence read. Short contigs and scaffolds (>149 bp but < 250 bp) were only
20 accepted when the average coverage was > 10. This step removed a further 95,417
21 entries from the reference. A total of 86,290 entries in the initial assembly that
22 related to 25,864 loci were processed by CAP3, generating 26,172 contigs that
23 related to 25,864 loci. All of these assembled contigs were combined with the
24 remainder to generate a consolidated quality filtered assembly of 168,046 contigs
25 and scaffolds.

1 The quality filtered data set was then compared to the Uniref90 database
 2 using BLASTX. An arbitrary E-value threshold of $<10^{-10}$ was applied for matches to
 3 protein sequences in the database, and 60,278 contigs and scaffolds met this
 4 criterion. The matched sequences were then further filtered, and any match that was
 5 not to a plant species was removed (11,565) leaving a final filtered reference set of
 6 48,713 contigs and scaffolds, representing a core transcriptome for perennial
 7 ryegrass. Sequences were further gap-filled if possible, generating a total length of
 8 70,192,231 bp, with a G/C content of 50.81%. The N50 statistic identified 9,610
 9 sequences of ≥ 2324 bp. Full details of the RNA-Seq data from this study can be
 10 accessed from NCBI BioProject PRJNA379202.

11

12 **Discussion**

13 Annotation of the present and any future draft genome assemblies for perennial
 14 ryegrass will be assisted by provision of high-quality reference transcriptome data, in
 15 order to increase confidence in gene prediction. In addition, alignment of perennial
 16 ryegrass coding sequences to putative orthologues in the genomes of model
 17 Poaceae species such as rice (*Oryza sativa* L.) and *Brachypodium distachyon* L.
 18 permits inference of conserved syntenic relationships, in support the broad patterns
 19 of conserved macrosynteny that have been previously established (Jones et al.
 20 2002; Pfeifer et al. 2013). Reference transcriptome assembly is also desirable for
 21 implementation of GBS methods based on sampling of transcription products as a
 22 means for genome complexity reduction (Harper et al., 2012). Such methods of likely
 23 to be of high value for species such as perennial ryegrass, due to large genome size
 24 and high levels of heterozygosity, which may lead to substantial amounts of missing
 25 data from GBS methods based on enzymatic cleavage of genomic DNA such as

1 restriction-associated DNA (RAD) systems (Miller et al. 2007). Transcripts
2 sequenced by RNA-Seq from specific genotypes may be aligned to the Impact04
3 reference transcriptome to identify polymorphic DNA sequences suitable for use in
4 applications such as genome-wide association studies (GWASs) (Huang and Han
5 2014) and genomic selection (Meuwissen et al. 2001; Newell and Jannink 2014).

6 A comprehensive transcriptome resource is also valuable for identification of
7 candidate genes for core biological functions in perennial ryegrass, such as variation
8 of flowering time, oligosaccharide biosynthesis and lignification processes
9 (Shinozuka et al. 2012). In particular, evaluation of the reproductive development-
10 specific libraries will assist the identification of candidate genes for the gametophytic
11 self-incompatibility loci of perennial ryegrass, controlled by the S and Z loci
12 (Shinozuka et al., 2010; Manzanares et al., 2016).

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Table 1.

Transcriptome sequence statistics by library of origin.

Tissue name – library identifier	Description of tissue source	Quality Sequence Reads Generated
Tip 1	Tip of the youngest leaf from a single vegetative tiller	82,057,679
Tip 2	Tip of the second youngest leaf from a single vegetative tiller	83,516,188
Tip 3	Tip of the third youngest leaf from a single vegetative tiller	67,954,787
Mid 1	Mid-section of the youngest leaf from a single vegetative tiller	71,535,548
Mid 2	Mid-section of the second youngest leaf from a single vegetative tiller	80,251,780
Mid 3	Mid-section of the third youngest leaf from a single vegetative tiller	76,357,990
Pseudo 1	Complete pseudostem from a single vegetative tiller	37,719,341
Pseudo 2	Lower portion of the pseudostem of a single vegetative tiller	40,605,629
Pseudo 3	Upper portion of the pseudostem of a single vegetative tiller	40,905,313
Root mid	Mid-section of root mass	39,663,070
Root tip	Tip section of root mass	40,204,367
Flower	Complete flower, (un)opened	32,437,273
Pollinated pistil 5 mins	Self-derived pollen added to pistil, then after 5 minutes pistil excised and frozen	34,693,932
Pollinated pistil 1 hour minus	Self-derived pollen added to pistil, then after c. 1 hour pistil excised and frozen	34,670,395
Pollinated Pistil 1 hour plus	Self-derived pollen already added to pistil, upon tissue harvest, for an undefined time greater than 1 hour prior to pistil being excised and frozen	41,587,233
Stigma pollinated 0mins	Harvested following pollination at 0 minutes	36,937,075
Stigma pollinated 5mins	Harvested following pollination at 5 minutes	43,747,723
Pistil	Whole pistil	23,111,505
Anther	Whole anther	23,590,397