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Identification of universal grass genes and estimates of their monocot-/ commelinid-/ grass-specificity

Rowan A. Mitchell[1, 2*]

[1]Rothamsted Research, United Kingdom, [2]Independent researcher, United Kingdom

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Scope Statement

The manuscript describes a novel bioinformatics pipeline that aims to identify all universal protein-coding genes in grasses. It does this using genomes, gene models, and ortholog tables for 16 grass species from the Ensembl Plants major plant bioinformatics resource. Genes are organised into groups to optimise HHM profile scores and novel gene models are discovered in genomes using these profiles. Specificity to monocots / grasses is defined based on best hits to profiles from non-grass species. Users can access the results from supplementary tables and large datasets made available online.

Conflict of interest statement

The authors declare a potential conflict of interest and state it below

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Abstract

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The evolutionary success of grasses is due to characteristics of resilience and fast growth in open habitats that led to their underpinning of agriculture and is attributable to many grassspecific traits. Genes responsible for these traits are likely specific to grasses, highly conserved and present in all grasses (universal genes) as they perform essential functions for fitness. A bioinformatics pipeline was developed to identify such genes using 16 grass full genomes in Ensembl Plants release 56. The first steps used existing gene models to generate groups of grass orthologs to rice and maize genes present in most grass species and refined membership of these groups such as to optimise the Hidden Markov Model (HMM) profile score from the HMMER package. These were then supplemented using new gene models found in grass genomes with the genBlastG tool; this step increased the number of universal groups by >2-fold to give 12,855 highly conserved, universal groups. Specificity for these groups was assessed using closest matching gene models from nonmonocot species. Possible cut-off values were tested with sets of known genes expected to be either of common function for all plants, or of commelinid-/ grass-specific function. A specificity metric based on HMM score from grass group profiles performed better than % identity as a means of discriminating between these common and specific function test sets.Using an appropriate cut-off for this metric, 5,701 of the groups were identified as monocot-/ commelinid-/ grass-specific of which 72% appeared to be grass specific. These results comprise the universal_grass_peps database available at DOI doi.org/10.23637/rothamsted.98ywz. This database can be searched by researchers to determine whether their experimentally identified grass genes match universal groups and, for those that do, to obtain systematic estimates of monocot-/ commelinid-/ grassspecificity.

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Identification of universal grass genes and estimates of their monocot- / commelinid- / grass-specificity.

Rowan A. C. Mitchell

rowan.mitchell@rothamsted.ac.uk

Rothamsted Research, Harpenden, Hertfordshire, AL5 2JQ, U.K.

https://www.rowanmitchell-grassscience.co.uk/

Abstract

The evolutionary success of grasses is due to characteristics of resilience and fast growth in open habitats that led to their underpinning of agriculture and is attributable to many grass-specific traits. Genes responsible for these traits are likely specific to grasses, highly conserved and present in all grasses (universal genes) as they perform essential functions for fitness. A bioinformatics pipeline was developed to identify such genes using 16 grass full genomes in Ensembl Plants release 56. The first steps used existing gene models to generate groups of grass orthologs to rice and maize genes present in most grass species and refined membership of these groups such as to optimise the Hidden Markov Model (HMM) profile score from the HMMER package. These were then supplemented using new gene models found in grass genomes with the genBlastG tool; this step increased the number of universal groups by >2-fold to give 12,855 highly conserved, universal groups. Specificity for these groups was assessed using closest matching gene models from non-monocot species. Possible cut-off values were tested with sets of known genes expected to be either of common function for all plants, or of commelinid- / grass-specific function. A specificity metric based on HMM score from grass group profiles performed better than % identity as a means of discriminating between these common and specific function test sets. Using an appropriate cut-off for this metric, 5,701 of the groups were identified as monocot- / commelinid- / grass-specific of which 72% appeared to be grass specific. These results comprise the universal_grass_peps database available at DOI doi.org/10.23637/rothamsted.98ywz. This database can be searched by researchers to determine whether their experimentally identified grass genes match universal groups and, for those that do, to obtain systematic estimates of monocot- / commelinid- / grass-specificity.

Introduction

Grasses (Poaceae) are of huge ecological importance, dominating open habitats in which they played a fundamental role in forming (Jacobs et al., 1999;Kellogg, 2001) such that this group of organisms now covers one third of global land area. Indeed it has been suggested that the rise of the grasses some 40 MYA was a key event in earth history, changing the water cycle, carbon cycle and climate permanently (Retallack, 2001). Their adaptation to open habitats has made them suited to adoption in agriculture and all the origins of human civilisation are associated with domestication of cereals and/or of grazing animals. Today, about 70% of the calorie intake for humans comes directly or indirectly from grasses (FAOSTAT, 2019).

Grasses co-evolved with large grazing mammals which few other plants can withstand during early growth giving rise to the open grassland habitats (Stebbins, 1981). Key grass adaptations to this ecosystem include: morphology that allows meristems to avoid consumption and fire damage allowing regrowth; tissues rich in silica to resist herbivory and stress (Mitani-Ueno and Ma, 2021); stomata that can respond faster than those of other plants to rapidly changing conditions of open habitats (Chen et al., 2017); cell walls containing ferulate implicated in lowering digestibility and stress resistance (Chandrakanth et al., 2023); unique inflorescence and seed characteristics for efficient reproduction (Kellogg, 2001). These traits are the result of specific protein-coding genes, non-coding genes and regulatory genomic elements that arose in the evolution of grasses; here my aim was to develop a pipeline to identify the protein-coding genes (henceforth referred to as “genes” for brevity) involved in grass-specific traits.

Relatively few genes involved in these traits have been demonstrated experimentally (examples listed in Table 1). Among the best characterised are Lsi1, Lsi2 and Lsi6 genes encoding silicic acid transporters that are required for Si accumulation and distribution. Both monocots and dicots have homologs of Lsi1 and Lsi2 that transport silicic acid, but Lsi1

differs in polarity and localisation in monocots; monocots additionally have Lsi6 transporters that direct Si transport within nodes (Ma and Yamaji, 2015;Mitani-Ueno and Ma, 2021). Grass cell walls differ from those in dicots in several respects and genes responsible for the grass-specific features are now known for: presence of (1,3;1,4)-beta-D-glucan (Burton et al., 2006), ferulate moieties on the polysaccharide arabinoxylan that can cross-link xylan chains or xylan to lignin (Feijao et al., 2022;Chandrakanth et al., 2023), lignin monomer tricin (Lam et al., 2015) and beta-expansins which specifically mediate expansion of the differently composed grass primary cell walls (Sampedro et al., 2015). Numerous monocot-specific regulatory genes implicated in determining the unique morphology of grass inflorescence have been identified; some of best characterised are the ramosa2 (Bortiri et al., 2006) and LOFSEP transcription factors (Kobayashi et al., 2012;Wu et al., 2018). Finally, some genes involved in the fast-responding grass stomata such as guard cell SLAC1 anion channel have been experimentally described (Schäfer et al., 2018).

I postulated that these grass genes and others responsible for functions specific to monocots / grasses that are key to grass fitness will be (1) present in all grasses i.e. universal, (2) highly conserved (3) have no close homologs in species outside monocots. The concept of universality of genes – matching genes being present in all organisms within a taxonomic unit - is a useful guide to their importance for fitness and implicitly groups genes by function (Kriventseva et al., 2018). On point (3), it is convenient to consider monocot- and grass-specificity together because the large number of non-monocot plant genomes and wealth of gene knowledge (particularly for Arabidopsis) make for a better reference set than the few, less studied non-grass monocot genomes. Also many key gene functions may have evolved first in monocots and then been expanded by gene duplication in grasses. Thus the aim is to capture those genes with key functional innovations that arose in monocots or grasses and have not diverged further within the grasses; from typical estimates of timescales for origin of

monocots and divergence of grasses (Bouchenak-Khelladi, 2007), these function innovations would have occurred in the period between 150 and 50 MYA.

The likelihood that two genes from different species share the same function increases with the similarity of the encoded peptide sequences. For a given level of sequence similarity, it is thought that they are more likely to share function if they are orthologs, i.e. descended from the same gene in the common ancestor (Gabaldón and Koonin, 2013). Here I also assumed that universality of genes, i.e. if similar genes are found in every species of a taxonomic unit, can also be taken as supporting common function as it may imply a role in a trait essential for fitness. Furthermore, using this set of genes of putative common function allows the use of profiles that emphasise the conserved sequence elements that are key to that function rather than weighting the whole sequence equally.

I used these principles to design the novel bioinformatics pipeline described here which aims to: (a) identify a maximal set of groups of highly similar genes found in all grasses with each group having putative common function (b) assign estimates of how specific these functions are to monocots / commelinid- / grass species based on closest hits from species outside these taxa. Using the set of the genes described above to define a cut-off for specificity, groups were classified as having monocot- / commelinid- / grass-specific or non-specific functions. I report some of the characteristics of these specific gene sets. Finally I discuss uses and limitations of these predictions.

Methods

Predefined gene sets

To help test the pipeline output and to select cut-off values two sets of genes were pre-defined. The small number of monocot- /commelinid- / grass specific protein-coding genes of known function (Table 1) were used as a specific test set. A list of proteins of known function expected to be common across all plants was also compiled to act as the non-specific test set. This non-specific set was derived from ribosomal subunit proteins using RPG database (Nakao et al., 2004) (http://ribosome.med.miyazaki-u.ac.jp) and enzymes or enzyme subunits in amino acid synthesis, glycolysis, photosynthetic electron transport, CBH cycle and nucleotide synthesis from OryzaCyc database which had identical steps in AraCyc database within Plant Metabolic Network (Hawkins et al., 2021) giving a total of 240 rice peptides (Table S1).

Pipeline overview

Figure 1 shows a scheme of the pipeline which takes input data downloaded from Ensembl Plants, processes these using custom software and public packages and generates datasets that populate a novel database called universal_grass_peps.

The following input data were manually downloaded from the Ensembl Plants database (Bolser et al., 2016) release 56 (https://feb2023-plants.ensembl.org/): peptide sequences (peps) from gene models for 16 grass species and 58 non-grass species (Table S2), the full genome sequences of the grasses with their gene annotations, and the ortholog tables of rice and maize gene models to all other grasses (downloaded using the Biomart tool). Rice and maize were chosen as the reference species because they are intensively studied crops with well annotated genomes representing respectively the BEP and PACMAD clades that together include all grasses.

All operations on these input data were carried out on the Rothamsted Linux High Performance Cluster using custom Perl (with Bioperl routines; Stajich et al., 2002) and bash scripts to run bioinformatics tools and process data. These scripts are available at https://github.com/Rowan-ACM/universal_grass_peps. The complete pipeline took 11 days of run time on the cluster to complete.

The methods used in the different components of the pipeline shown in Fig. 1 are described below.

Identification of highly conserved peptides present in all grasses (Find Universal Groups block in Fig. 1)

Using peps from gene models of the 16 grass species, any identical ones were removed but all non-identical peps from splice variants were retained (for convenience, “gene” is used here to mean a unit encoding a unique peptide). For the rice and maize reference species, using BLAST+ package (Camacho et al., 2009) a blastp search (parameters: -evalue 1.e-5 -max_target_seqs 50 -seg no -max_hsps 1) of peps was conducted against all others within same species and defined clusters where peps are >90% identical in both directions of a pairwise comparison for all comparisons within a cluster. Out of 40,196 rice peptides, 6% were in clusters with >1 member, mostly highly similar splice variants. In maize 37% out of 62,559 peps were in such clusters; this higher percentage is expected in maize due to the recent whole genome duplication (Swigonova et al., 2004) and greater propensity for tandem duplications (Guo et al., 2019). An ortholog table from the Ensembl multiple tables was defined where each entry was defined by a primary key (group ID) of the rice peptide or peps cluster ID. Where there was no maize ortholog, the most similar maize pep was found with blastp and if this was not orthologous to another rice gene, added all the other grass genes orthologs of the maize gene to the group and group ID was set to composite of rice and maize seed cluster IDs. Groups from maize were also allowed where there was no rice ortholog or similar pep sequence (as this could be added by the later genBlastG step). Other

grass species orthologs to rice and/or maize were assigned exclusively to a single group based on highest ranking by Ensembl ortholog confidence flag (0 or 1; defined from tree-compliance and, in a small number of cases, whole-genome alignment and gene order conservation; https://plants.ensembl.org/info/genome/compara/peptide_compara.html) then sequence similarity. Groups which had entries for fewer than 12 of the 16 grasses were deleted and genes orthologous to remaining groups were reassigned according to this ranking. At this stage (Box 1 Fig. 1) there were groups of multiple genes per grass all of which were classed orthologous to rice and/or maize. Using two reference species in this way allows for similar non-orthologous genes of potentially common function to be grouped together due to descending from two paralogs. But by using the ortholog information orthologous peps were more likely to be assigned to same group than non-orthologs with same level of similarity. This is designed to help to group by function in accordance with the principle that orthologs are more likely to share function at a given level of sequence similarity (Gabaldón and Koonin, 2013).

The next step (box 2 Fig. 1) was to optimise membership of groups keeping only one peptide sequence per species. This approach makes the profile scores comparable across all groups and avoids biasing profile to species with many members in a group. HMM profiles of each group were initially generated using the top ranked pep sequence for each species. To make HMM profiles, all the group member peps were aligned using MUSCLE v3.8.1551 with default parameters (Edgar, 2004) then the HMM profile was generated from this multiple alignment with hmmbuild (parameters --amino --fragthresh 0) and hmmpress commands from HMMER package version 3.3.2, Nov 2020 (Eddy, 2022). Similarity scores of the member sequences against their own profile were obtained using hmmscan (all hmmscan steps in pipeline used parameter E 1.e-7, other parameters default). To compare across groups, this score was normalised to a maximum possible score obtained with the consensus sequence of the profile (generated by hmmemit command) as the query to derive a HMM relative score (R). Then group members were each substituted with all the

alternative peptide sequences for this group and species; if R was improved by > 0.01 the substitution was kept as the group member; this requirement means that peptide sequences ranked as best orthologs in previous step tended to be kept as group members. It was found that groups could be further improved by using grass Ensembl gene models hits to the HMM profile found with hmmscan that were not members of other groups; these are peps not found by previous steps probably because they were not in ortholog tables. Again, these peps were assigned as group members if they improved R by >0.01 (box 3 Fig. 1).

In the next step (box 4 in Fig. 1) the genBlastG tool was used (She et al., 2011) which searches for gene models with canonical splice junctions in genomic sequence using a query peptide sequence; here the consensus from HMM profile for the group was used as the query. For each group, and for each grass where the current member was missing or low scoring, the relevant grass genome was searched with genBlastG (v138, parameters -p genblastg -v 2 -h 0 -j 3 -r 1 -norepair). Any hits discovered by genBlastG were checked that they were novel by comparing exon coordinates with those of all Ensembl gene models using gff files. Using criteria as above, if a novel gene model from genBlastG improved the profile, it was adopted as the group member for that species and the HMM profile was rebuilt. A maximum of 4 genBlastG gene models were adopted so every profile has at least 12 Ensembl gene models. At the completion of this process, the R value was recalculated for each member and groups where the lowest scoring member had R < 0.65 or had missing members were discarded; the cut-off of 0.65 is a criterion for high conservation and the value was selected as that for which 90% of the pre-defined expected universal non-specific genes (Table S1) groups passed. HMM profiles from the complete set of groups that pass these were compiled into a single HMMER database, the universal_grass_peps HMM database.

Matches of grass genes to universal groups (box 5 in Fig. 1)

All scores for Ensembl grass peps against the universal_grass_peps HMM database for all groups were obtained. All non-members that had scores of R > 0.65 to any group were allocated as associate peptides allowing many-to-many relationships (this allows a lookup search with any peptide ID as query to find all groups to which a peptide is similar). To check whether some universal_grass_peps groups can be regarded as likely same function, the R of grass group members against other group HMMs were obtained. Where all members of a group scored > 0.65 for another group and *vice versa* these groups were allocated to a supergroup of potential same function.

Monocot- / Commelinid- / Grass- Specificity (Estimate Specificity block in Fig. 1)

Scores were obtained for the best-matching non grass peptide sequence from all the 58 non grass species against universal_grass_peps HMM database for all groups. A metric of specificity S for each group was evaluated, defined as R of the lowest scoring grass member of this group minus R of highest scoring non-monocot peptide. By definition a value of S <= 0 means the non-monocot peptide scores highly enough to be included so the group is completely non-specific.

Different cut-off values for this threshold were investigated using the groups containing the genes from the pre-defined specific or non-specific test sets. For comparison of the S metric with simple pairwise percentage identity, this was calculated from global alignment by MUSCLE of the rice member of the group to its closest non-monocot hit identified by blastp.

Functional annotation of monocot- / commelinid- / grass-specific groups

To characterise the functions of the set of groups classified by the pipeline as monocot- / commelinid- / grass-specific groups, functional annotations were obtained.

General gene descriptors and Gene Ontology terms from Ensembl Plants were assigned to groups from their member rice and maize peps. Where present, linked publications, gene

240 descriptors and symbols and trait ontology were assigned to groups from database entries
241 for their member peps taken from RAP-DB (Sakai et al., 2013) and KnetMiner-rice for rice
242 and MaizeMine (Shamimuzzaman et al., 2020) for maize and KnetMiner -wheat for wheat.
243 Entries were retrieved from web interfaces except for KnetMiner where cereals knowledge
244 graph (Hassani-Pak et al., 2021) with programmatic access was used to retrieve gene-TO
245 and gene-GO relations for wheat and rice genes along with supporting publications.

246

Results

Identification of highly conserved peptides present in all grasses (Find Universal Groups block in Fig. 1).

Initial steps (boxes 1-3 in Fig. 1) identified 17,816 groups of similar peps that were present in at least 12 of the 16 grass species from their original gene models present in Ensembl Plants release 56. Of these, 6,354 groups passed criteria for universality and high conservation (i.e. had members for all 16 species and minimum R > 0.65). However, correct gene models are frequently missing from annotated genomes particularly where there is no transcript information to support these as is often the case for lower expressed genes in less well studied species. Therefore the genomic sequences were searched for gene models for each group and for each gene model that was missing or low scoring using the genBlastG tool with consensus peptide sequence of the group HMM profile as query (box 4 in Fig. 1). By incorporating the new gene models identified into groups the number of highly-conserved universal groups was more than doubled from 6,354 to 12,855 showing the importance of the genBlastG step. The species break-down of the new gene models obtained by genBlastG (Table 2) within these groups shows the newer genomes from *Saccharum spontaneum* and *Lolium perenne* have the most whereas the intensively studied wheat with extensive transcript resources has the fewest.

The results for universal groups can be compared with those from the OrthoDB database which allows users to select for ortholog groups that are present in a minimum number of species (Kriventseva et al., 2018). At the Poales level in OrthoDB release 10 there are 2,581 ortholog groups that are present in all 11 grass species and there is substantial overlap with the groups here with 85% of rice RABP IDs are present in universal groups before the filter for high conservation. At this stage far more universal groups were recovered than from OrthoDB and this seems to be mostly due to the genBlastG step rather than relying on existing gene models.

The set of 12,855 highly conserved, universal groups obtained from the above steps are here termed the universal_grass_peps database. These groups contain sequences that all match the profile well but also contain different degrees of divergence. Two example multiple alignments used to generate the HMMs for two groups are shown in Figure 2. These show high conservation including for the novel genBlastG gene models but also reveal some of the inherent complexities found in most profiles; group Os03t0786600-01 has overwhelmingly similar sequences but also has some signs of divergence at the C-terminal between BEP (species 1-8) and PACMAD clade grasses (species 9-16), and group Os02t0763000-01 has a section found only in one species. Nevertheless these alignments do support the hypothesis of highly similar function common to all grasses for these groups.

Matches of grass genes to universal groups (box 5 in Fig. 1)

All grass gene model peps were searched against the HMM profiles of universal_grass_peps for hits with R above the cut-off of 0.65; if these are not the member of any group they are classified as associated to the group. The total number of associated peps for each species is shown in Table 2 and generally reflects the degree of gene duplication. The grass pep hits are also used to define supergroups; if all members of one group are hits above cut-off to another group, the two groups are assigned to super-groups of closely related function. A total of 799 supergroups were identified (Table S3). Supergroups can contain groups with same molecular function but differing regulation due to sub-functionalisation.

Monocot- / Commelinid- / Grass- Specificity (Estimate Specificity block in Fig. 1)

All peps from the 58 non-grass species in Ensembl Plants were scored against the HMM profiles of universal_grass_peps. The results were used to derive the specificity metric S for

each group, where S is minimum R value from group members minus maximum R value for any peptide from non-grass to give monocot-/ commelinid-/ grass-specificity. Distinguishing between monocot-specificity, commelinid-specificity and grass-specificity is dependent on maximum R values from only 3 species (two non-grass commelinids and one non-commelinid monocot) so these sub-classifications are less secure, and the overall monocot-/ commelinid-/ grass-specificity is emphasised here.

The S metric is a measure of sequence divergence from the grass profile that can be used as a basis for an initial hypothesis of function divergence in the same way that other sequence-based measures are used. The pre-defined test sets were used to gauge the performance of S as a means of determining specificity, i.e. the non-specific test set of 215 peps expected to have common function in all plants because the fundamental processes they are responsible for are not thought to have diverged (Table S1) and the specific test set of 16 peps with monocot- / commelinid-/ grass-specific functions (Table 1). The S metric was compared with simple pairwise % identity with the best non-monocot hit for these sets (Figure 3); S performs better than % identity at discriminating between the two sets as choosing highest cut-off with no false negatives gives 11.6% false positives using S and 14.4% false positives using pairwise percentage identity. Using sequence similarity (e.g. from BLOSUM62) rather than identity did not improve performance of pairwise alignment as a measure (data not shown).

Applying the cut-off S of >0.25 which gave 11.6% false positive and no false negatives with the test sets (Figure 3) to the complete set of 12,855 groups gave 5,701 defined as monocot- / commelinid- / grass-specific. This set was divided into subsets classified as probably monocot-specific (355 profiles), commelinid-specific (1,260 profiles) and grass-specific (4,086 profiles) based on values of S calculated from best hits for each taxonomic level and is listed in Table S4.

Functional annotation of monocot- / commelinid- / grass-specific groups

Functional annotations for these 5,701 specific groups were derived from public annotations of their rice, wheat, and maize members. Most (~90%) have no linked publications and only general descriptors and high-level GO terms based on domains. When the set is ranked by S metric, the groups with least similarity to any non-grass pep often have nothing known but a prominent domain is “Cyclin-like F-box domain” which occurs in 4 of the 20 most grass-specific profiles (Table S4). Proteins containing this domain were also highlighted in an early study attempting to identify grass-specific proteins (Campbell et al., 2007). F-box domains are associated with protein-protein interaction e.g. for the regulation of other proteins by ubiquitination.

A wider view of the processes in which the monocot- / commelinid- / grass-specific genes take part can be gained from analysis of GO terms assigned in RAP-DB and MaizeMine, based mostly on recognition of domains and functions of homologs. Of all the biological process GO annotations, most are assigned to at least one group suggesting there are some monocot- / commelinid- / grass-specific aspects of most processes in grasses. The processes that are dominated by these specific genes are shown by the terms which are enriched; there is clear enrichment of groups of regulatory proteins, especially those involved in control of transcription and of protein activity (Table 3). Some specific enriched terms include ones that might be expected such as xylan biosynthesis and leaf development but also include fundamental processes such as cell cycle. Enriched molecular function GO terms are mostly DNA-binding and enzyme activities; the most enriched enzyme category is hydroxycinnamoyl transferase activity (Table 3) which may reflect the importance of these moities on lignin and xylan polymers in grass cell walls (Chandrakanth et al., 2023).

For the minority of groups with associated publications, the publications, gene descriptors and trait ontology (TO) terms from the RAP-DB, MaizeMine and KnetMiner databases were assigned. The traits defined by TO terms are associated with variants of the member rice,

maize and/or wheat genes from evidence in the publications. For the monocot- / commelinid- / grass-specific groups (Table S4), particularly common traits affected are grain size (90 groups), flowering time (62 groups), with numerous morphology traits as might be expected. However also common are traits for insect / pathogen defence and abiotic stress resistance.

The complete set of 5,701 groups defined as monocot- / commelinid- / grass-specific together with specificity estimates and all functional annotation are in Table S4.

Discussion

Genes of common function that occur in all species within a taxonomic unit (universality) indicate that the function is likely key to fitness. Although sequence similarity is a measure of likelihood of shared function, using existing bioinformatic resources it is not straightforward to compare genes in a systematic way, nor to check for criterion of universality given variation in completeness of genome annotation. The new approach described here provides predictions of all universal grass genes with putative common function and estimates of their specificity to monocots / commelinids / grasses. It should be noted that since the pipeline generates groups with putative common function that can contain any similar gene, not just true orthologs, it is not directly comparable to software like OrthoFinder that identify orthologous groups (Emms and Kelly, 2019). Rather, ortholog tables are an input to the pipeline as a starting point for seeding groups (box 1 in Fig. 1) but these predicted orthologs can be replaced in a later step by alternative peps from the same species if they match the profile better (box 3 in Fig. 1). A novel aspect of the pipeline is the emphasis on universality which led to the incorporation of the genBlastG step to find missing genes (box 4 in Fig. 1); this step generated 14,038 new gene models. The fact that grass species like wheat that have more RNAseq data require fewer of these gene models (Table 2) suggests that future grass RNAseq studies will validate many of them. The use of a metric based on HMM profile score to estimate how specific the function of a universal group is to monocot / commelinid / grass species is another novel aspect of the pipeline; it provides a systematic basis for an assertion of such specificity for genes of unknown function.

Importance of monocot- / commelinid- / grass-specific genes

Grasses typically have a haploid set of about 40,000 protein-coding genes. The analysis here indicates that about 12,000 of these are universal in grasses and that about half of universal genes are monocot- / commelinid- / grass-specific. These genes are enriched for

regulatory functions (Table 3) as might be expected given the radically different organisation and morphology of grasses. The great majority of genes in the specific sets are of unknown function which reflects our lack of understanding of molecular mechanisms underlying grass-specific characteristics. However, the GO term annotation indicates that these genes are likely involved in virtually every process in grasses and are particularly dominant in the enriched ones shown in Table 3 which include cell wall processes and stomatal regulation as might be predicted but also some less expected such as control of epigenetic marks and chloroplast movement.

The importance of variants of the monocot- / commelinid- / grass-specific genes for crop traits is seen from publications associated to the identified sets (Table S4) including numerous variants associated with grain yield, abiotic stress and defence. Where a trait is known to be commelinid- or grass-specific, the classifications generated here can help to identify candidate genes involved in the trait. In our own work on dietary fibre QTLs in wheat grain, candidate genes identified as likely commelinid- / grass-specific were prioritised as dietary fibre is mostly feruloylated arabinoxylan (AX) that only occurs in commelinid species. The causal allele was eventually shown to be a variant of one such gene – a commelind-specific peroxidase involved in cross-linking AX (Mitchell et al., 2023). There must be many more valuable natural and induced variants of these genes yet to be discovered and the classifications generated here could help in candidate identification.

Limitations of approach

All high-throughput predictions of shared function based almost entirely on peptide sequence need to be used with caution and cannot substitute for detailed knowledge of the particular protein. The approach here should be treated as a first best guess of shared function similar to comparing percentage identity (as biologists often do as a first step) but more likely to be accurate (Fig. 3) as the HMM approach weights the conserved parts of sequence important for function, exploiting the fact that the identified genes are highly conserved and present in

all grasses. The gene groups would be expected to include nearly all cases of genes which have identical function in all grasses, but they can also include cases where there are highly similar functions with divergent aspects. This is because non-conserved regions do not affect the profile score much so if there is a conserved core and divergent, species-specific functional aspects of the sequence they can still pass the highly conserved filter. Therefore the next step after identifying a group of interest based on its S score should be to inspect the multiple alignment (as in Fig. 2) to judge the extent of divergence in different grasses; all 12,855 multiple sequence alignment files are available in the universal_grass_peps database.

Too much divergence from the group profile will lead to the group being excluded. These cases will likely include genes that were important in evolutionary history of grasses but have subsequently diverged in adaptation to the many different ecosystems that grasses occupy since their divergence some 55 MYA (Bouchenak-Khelladi, 2007) including the major bifurcation into the BEP and PACMAD clades with respectively C3 and C4 photosynthesis.

Uses of universal_grass_peps database

Where experiments reveal large sets of grass genes or peps such as transcriptomics, proteomics or genes underlying QTLs, they are inevitably dominated by genes with little or no information on function. Even for rice, probably the most studied grass, only 13% of genes in RAP-DB database (Sakai et al., 2013) have associated publications and only a minority of these publications specify function. For such unknown genes it is useful to have a systematic approach to identifying those that are of grass- / commelinid- / monocot- specific function as this information can point to the nature of the process they are likely involved in. For example a network of co-regulated genes identified from transcriptomics enriched for grass-specific functions indicates involvement of the network in a grass-specific trait such as inflorescence development, Si deposition etc. Using the look-up tables generated, any set of

grass genes from the grass genomes used here can be used to find all those in, or associated to, the universal groups and their categorisations as likely monocot-, commelinid- or grass- specific. For other grass genes, the HMMs database for the universal groups can be searched using the HMMER package. For genes with matches in the universal groups, the value of the S metric is a measure of how different the group is from any non-grass pep and the supplied multiple alignments can be used to judge divergence from the profile.

The universal_grass_peps database is available at https://doi.org/10.23637/rothamsted.98ywz. On the top directory there is a user guide and summary spreadsheet of all 12,855 groups; the HMM database, multiple sequence alignments, genBlastG gene models and lookup tables for grass genes are in subdirectories.

Future developments

The pipeline reported here is a first attempt to implement the concept of using universal genes to identify groups of putative common function but could be improved upon with different software in future. Improvements might be made by using recently released alternative packages for finding orthologs (Emms and Kelly, 2019) as the first step (box 1 in Fig. 1) and gene models in genomes (Li, 2023) (to replace genBlastG for box 4 in Fig. 1) with reportedly better performance. Further in the future, two more major changes would be to use structural prediction and incorporate expression patterns. The use of HMMs is a convenient and fast way of obtaining profiles for groups against which other sequences can be scored for matches but here it is actually a proxy for comparison of structures. A direct comparison of predicted structures such as that generated by AlphaFold might be a better approach. Also similar expression patterns of peptides from different species are a strong indicator of shared function and would help resolve cases of sub-functionalisation (Das et al., 2016). However, I am not aware of any software packages capable of conveniently and

quantitatively comparing predicted structures or expression patterns that could be used to achieve these improvements in the pipeline at present.

Conclusion

A novel bioinformatics approach was used to try to identify all universal grass genes coding proteins responsible for monocot- / commelinid- / grass-specific traits, making the first estimates of the size of these sets. As part of this, 14,038 new gene models were generated for 16 grass genomes. The resulting classifications of grass genes can help interpretation of experimentally identified sets of grass genes and represent numerous gene research targets to improve our understanding of grass-specific mechanisms.

Tables

Table 1 Known monocot- /commelinid-/ grass-specific genes.

trait	gene family	gene name(s)	reference species gene ID(s)	description	reference
secondary metabolite, cell wall	cytochrome P450	CYP93G1; CYP75B4	Os04g0101400; Os10g0317900	production of tricin a secondary metabolite and lignin monomer specific to grasses / monocots	Lam et al., 2015
cell wall	cellulose synthase-like F	OsCslF2	Os07g0552800	makes (1,3;1,4)-beta-glucan, a component of grass cell walls absent in dicots	Burton et al., 2006
cell wall	BAHD acyl-CoA transferases	BAHD01 / AT9; BAHD05 / AT1	Os01g0185300; Os01g0615300	implicated in formation of feruloyl-arabinfuranosyl precursor prior to additon to xylan, a key feature of commelinid cell walls	Chandrakanth et al., 2023
cell wall	BAHD acyl-CoA transferases	PMT1; FMT / AT5	Os05g0136900; Os05g0278500	mediates addition of hydroxycinnamates to monolignols leading to commelinid-specific features on lignin	Chandrakanth et al., 2023
cell wall	glycosyl transferase family 61	XAX1	Os02g0329800	implicated in addition of feruloyl-arabinofuranose to xylan, a key feature of grass cell walls	Feijao et al., 2022
cell wall	expansins	EXPB9	Os10g0548600	grass-specific beta-expansins evolved to mediate expansion in grass primary cell walls	Sampedro et al., 2015
inflorescence morphology	MADS transcription factor	OsMADS1; OsMADS5	Os03g0215400; Os06g0162800	specifies spikelet identity in rice inflorescence	Wu et al., 2018
inflorescence morphology	MADS transcription factor	MADS34/PAP2	Os03g0753100	PAP2 / OsMADS34 regulator of spikelet identity. Controls developmental processes unique to grasses	Kobayashi et al., 2012
inflorescence morphology	LOB domain transcription factor	ramosa2	Zm00001eb123060	ramosa2 responsible for genetic control of grass-specific inflorescence	Bortiri et al., 2006
Si transport	MIP/aquaporin membrane proteins	LSi1	Os02g0745100	transporter required for active uptake of Si in grasses	Ma and Yamaji, 2015
Si transport	MIP/aquaporin membrane proteins	LSi6	Os06g0228200	transporter required to control distribution of Si to leaves and panicle in rice	Mitani-Ueno and Ma, 2022
stomata	S-type anion channel family	SLAC1	Os04g0574700	contains a monocot-specific motif that confers nitrate-sensitivity to guard cell anion channel	Schäfer et al., 2018

Table 2 Counts of peps or groups for each grass species in universal_grass_peps database

grass species	Group members*	Members that are genBlastG gene models	Groups with associate peps	Max associate peps in one group	Total associate peps
Brachypodium_distachyon	12,855	312	4,776	25	9,926
Hordeum_vulgare	12,855	899	2,934	33	6,185
Leersia_perrieri	12,855	1,317	4,375	15	8,128
Lolium_perenne	12,855	2,142	3,084	28	6,059
Oryza_rufipogon	12,855	921	4,278	16	8,151
Oryza_sativa	12,855	1,819	3,060	16	4,912
Secale_cereale	12,855	491	2,810	39	7,977
Triticum_aestivum	12,855	178	10,965	91	68,522
Echinochloa_crus-galli	12,855	287	10,752	48	39,486
Eragrostis_curvula	12,855	1,329	5,026	27	9,830
Panicum_hallii_HAL2	12,855	212	4,166	17	7,957
Saccharum_spontaneum	12,855	2,305	7,419	36	16,944
Setaria_italica	12,855	796	4,199	23	8,085
Setaria_viridis	12,855	131	4,886	22	10,183
Sorghum_bicolor	12,855	276	4,485	36	9,167
Zea_mays	12,855	623	7,854	23	22,744

*by definition, all grass species have same number of group members

Table 3 GO terms that are enriched in Ensembl annotation for rice and maize members of the monocot-/ commelinid-/ grass-specific universal groups. All GO term names that occur in at least 5 groups and are enriched relative to rice, maize peps and to universal peps >2-fold are shown.

	number of monocot-/commelinid-/grass-specific groups	enrichment relative to Os, Zm peps	enrichment relative to all grass_universal peps
GO Domain: biological_process			
positive regulation of DNA-templated transcription	62	2.5	2.3
cell differentiation	48	2.9	2.1
negative regulation of catalytic activity	27	2.8	2.2
regulation of cyclin-dependent protein serine/threonine kinase activity	18	2.5	2.0
response to endoplasmic reticulum stress	13	2.7	2.3
regulation of jasmonic acid mediated signaling pathway	12	2.6	2.7
interstrand cross-link repair	12	3.5	2.4
cellular response to nitrate	12	5.9	2.5
mitotic cell cycle phase transition	12	2.4	2.4
nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	12	2.1	2.1
positive regulation of transcription from RNA polymerase II promoter in response to heat stress	12	8.1	2.7
DNA-templated transcription termination	12	2.2	2.4
regulation of primary metabolic process	11	2.2	2.5
negative regulation of endopeptidase activity	11	2.2	2.7
xylan biosynthetic process	11	2.9	2.0
regulation of nitrogen compound metabolic process	11	5.8	2.5
gene silencing by RNA-directed DNA methylation	10	2.8	2.9
regulation of leaf development	10	6.0	2.1
plastid transcription	7	3.3	2.3
mRNA destabilization	7	3.3	2.9
purine nucleoside transmembrane transport	6	2.8	2.2
nuclear-transcribed mRNA catabolic process, exonucleolytic, 3'-5'	6	3.1	2.2
rRNA methylation	6	2.5	2.2
positive regulation of helicase activity	6	5.1	2.2
mitotic spindle assembly	5	3.2	2.1
male meiosis II	5	4.7	2.9
negative regulation of organ growth	5	3.1	2.9
positive regulation of defense response to bacterium	5	7.7	2.1
mitochondrial mRNA modification	5	2.2	2.1
piecemeal microautophagy of the nucleus	5	3.2	2.9

positive regulation of mitochondrial translation	5	5.6	2.5
asymmetric cell division	5	3.0	2.9
chloroplast avoidance movement	5	2.2	2.9
malate transport	5	2.9	2.1
chloroplast accumulation movement	5	2.1	2.5
post-transcriptional regulation of gene expression	5	2.0	2.1
protein localization	5	2.3	2.1
regulation of mitotic cell cycle	5	2.6	2.1
response to red or far red light	5	2.7	2.9
GO Domain: cellular_component			
chromosome, telomeric region	8	2.4	2.1
RNA polymerase II transcription regulator complex	8	4.1	2.9
nuclear microtubule	6	2.2	2.9
cell periphery	6	3.1	2.2
plastid-encoded plastid RNA polymerase complex	5	3.5	2.9
DNA polymerase III complex	5	3.0	2.9
histone acetyltransferase complex	5	2.4	2.5
GO Domain: molecular_function			
sequence-specific DNA binding	197	2.4	2.3
DNA-binding transcription factor activity, RNA polymerase II-specific	79	2.2	2.2
RNA polymerase II cis-regulatory region sequence-specific DNA binding	73	2.5	2.2
hydroxycinnamoyltransferase activity	30	4.5	2.2
DNA-binding transcription activator activity, RNA polymerase II-specific	30	3.3	2.5
enzyme inhibitor activity	22	2.4	2.5
quercetin 7-O-glucosyltransferase activity	21	2.6	2.1
quercetin 3-O-glucosyltransferase activity	21	2.6	2.1
pentosyltransferase activity	14	2.4	2.1
ubiquitin conjugating enzyme binding	12	3.1	2.1
pectinesterase inhibitor activity	11	3.0	2.4
DNA-binding transcription repressor activity, RNA polymerase II-specific	11	10.6	2.4
histone acetyltransferase activity	10	3.0	2.2
glutathione binding	9	3.4	2.3
histone methyltransferase activity	8	4.5	2.1
ribonuclease activity	8	2.2	2.3
5'-3' exodeoxyribonuclease activity	7	5.2	2.0
myosin XI tail binding	7	2.8	2.9
galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase activity	7	2.2	2.9
endo-1,4-beta-xylanase activity	7	3.7	2.0
double-stranded RNA binding	7	2.5	2.5
purine nucleoside transmembrane transporter activity	6	2.9	2.1
xylosyltransferase activity	6	3.4	2.5

electron transporter, transferring electrons within the cyclic electron transport pathway of photosynthesis activity	5	2.1	2.9
strictosidine synthase activity	5	2.2	2.0
sucrose transmembrane transporter activity	5	6.4	2.0
myosin binding	5	2.8	2.9
ionotropic glutamate receptor activity	5	3.9	2.0
RNA-DNA hybrid ribonuclease activity	5	3.2	2.4
NAD+ ADP-ribosyltransferase activity	5	2.1	2.0
histone H3-methyl-lysine-9 demethylase activity	5	2.6	2.9
translation activator activity	5	6.4	2.4
telomeric DNA binding	5	3.2	2.4

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Figure Legends

Figure 1 Pipeline that generates the database of highly conserved universal grass protein-coding genes and estimates of their monocot- / commelinid- / grass-specificity (universal_grass_peps). All the input data is taken from Ensembl Plants release 56 and the processing steps are carried out by custom scripts, using the external tools shown in blue text, to generate universal_grass_peps database.

Figure 2 Two example group multiple alignments from the universal_grass_peps set of groups. Sequences are from grass spp 1. *Brachypodium distachyon* 2. *Hordeum vulgare* 3. *Leersia perrieri* 4. *Lolium perenne* 5. *Oryza rufipogon* 6. *Oryza sativa* 7. *Secale cereale* 8. *Triticum aestivum* 9. *Echinochloa crus-galli* 10. *Eragrostis curvula* 11. *Panicum hallii* HAL2 12. *Saccharum spontaneum* 13. *Setaria italica* 14. *Setaria viridis* 15. *Sorghum bicolor* 16. *Zea mays*. Sequences predicted by genBlastG have names starting “genblast” others are Ensembl gene models. Max score is the score of the consensus against the HMM profile generated from the alignment.

Figure 3 Proportion of groups of pre-defined genes expected to be of non-specific function (blue line) or specific function for commelinid / grass species (red line) that pass varying cut-off thresholds for two metrics of specificity. Upper panel: percentage identity of closest non-monocot hit to rice member of group. Lower panel: S metric defined as lowest HMM relative score of group member minus the top relative score for a non-monocot hit. In both panels the limit which gives no false negatives and minimum false positives is shown.

Supplementary Data

Table S1. Pre-defined set of grass genes expected to be universal and of non-specific function.

Table S2. All plant genomes used from Ensembl Plants release 56.

Table S3. Supergroups: contains groups which are so similar that all members would pass cut-off to be in another group within supergroup.

Table S4. Set of 5,701 groups classified as monocot- / commelinid- / grass-specific with details of specificity estimates, group properties, members, and functional annotation.

Figure 1.JPEG

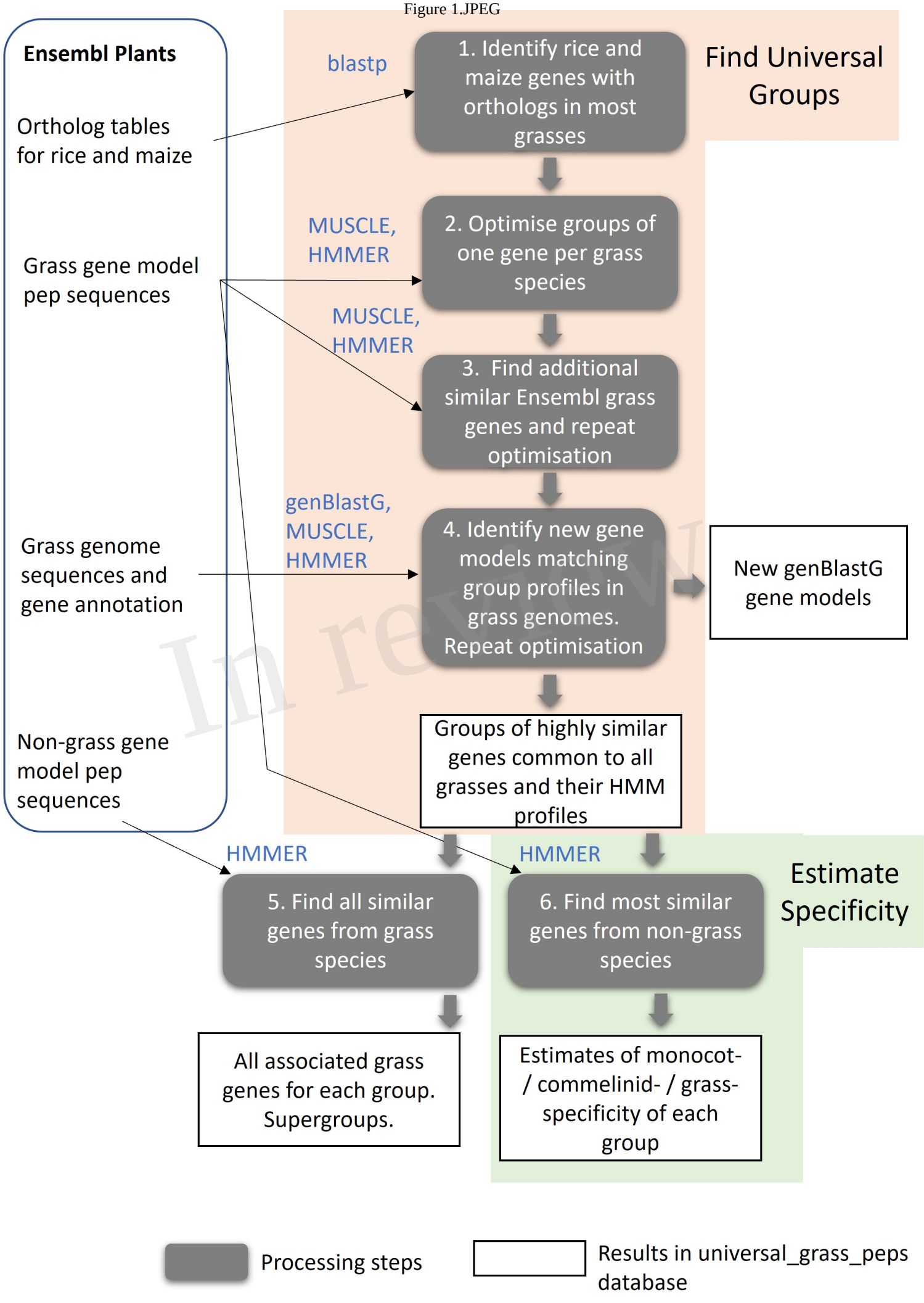
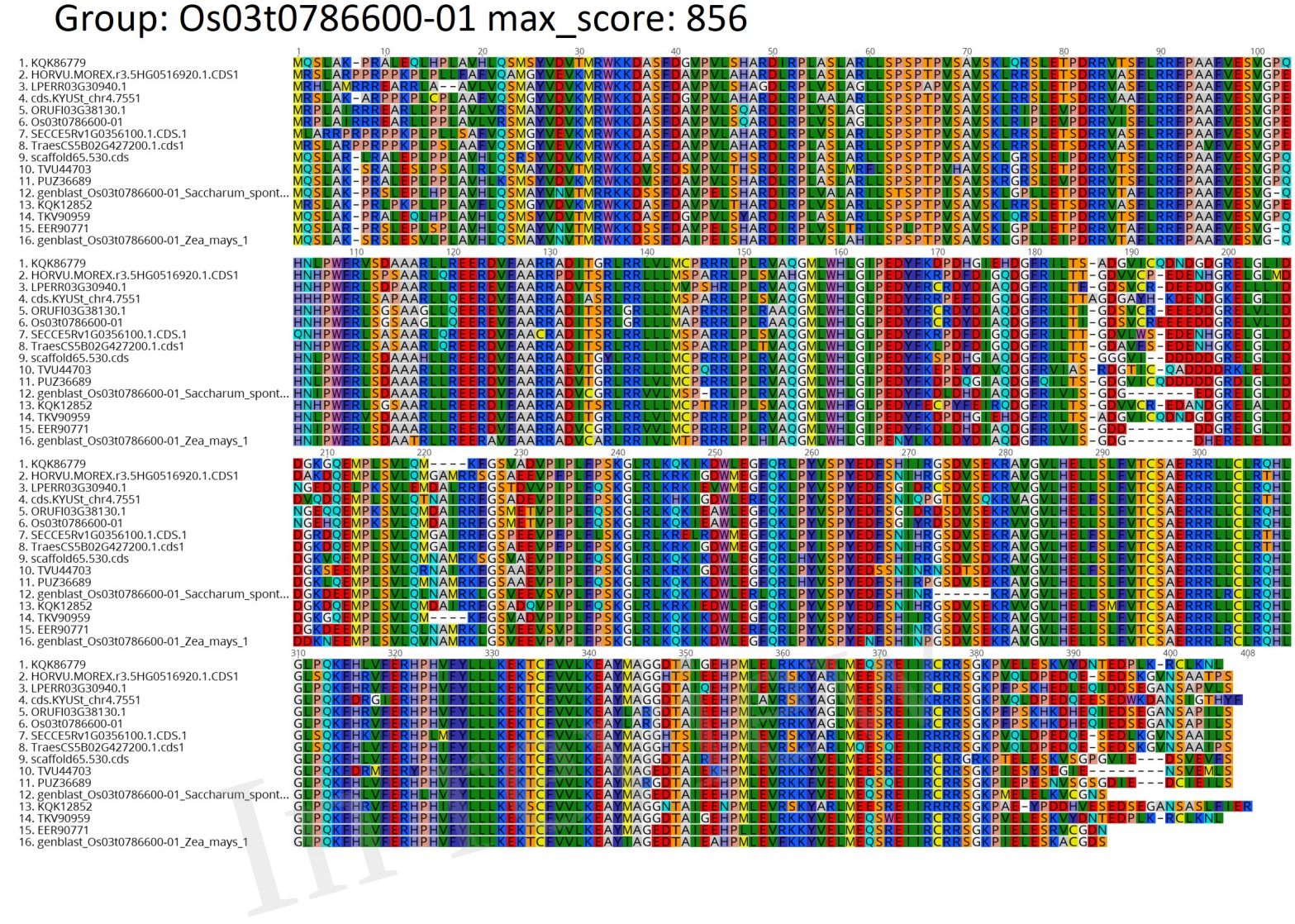


Figure 2.JPEG



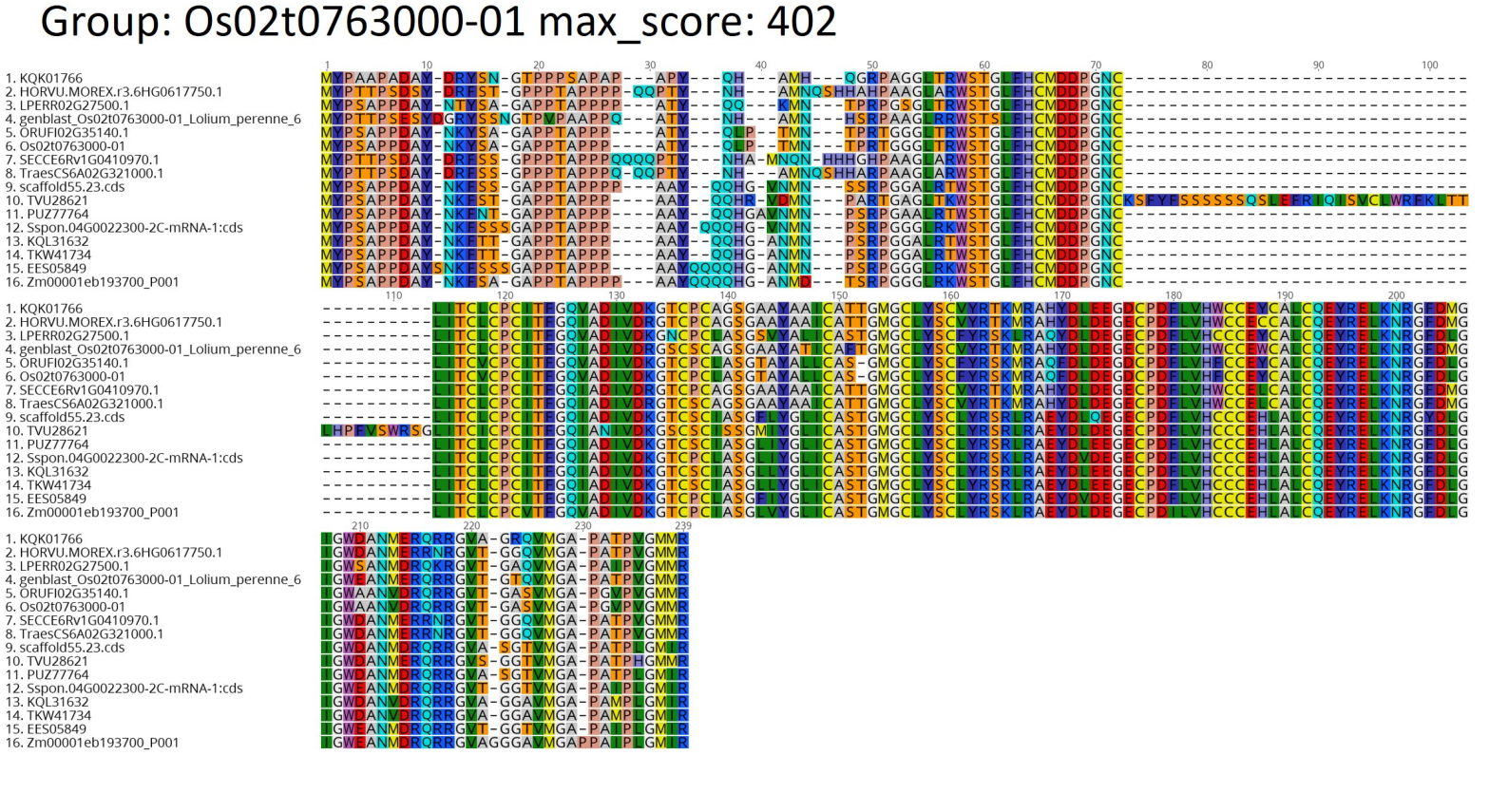


Figure 3.JPEG