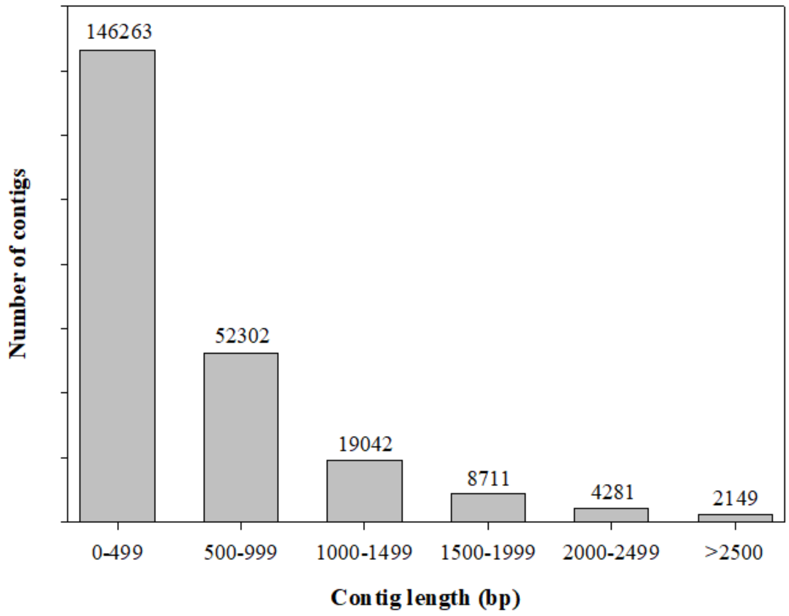
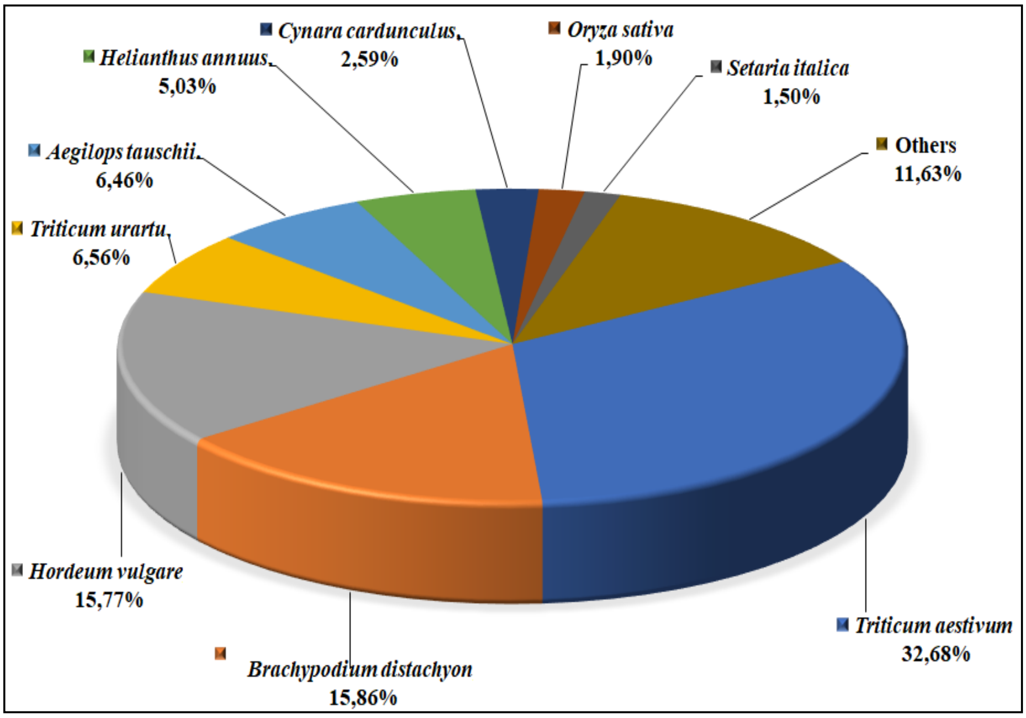
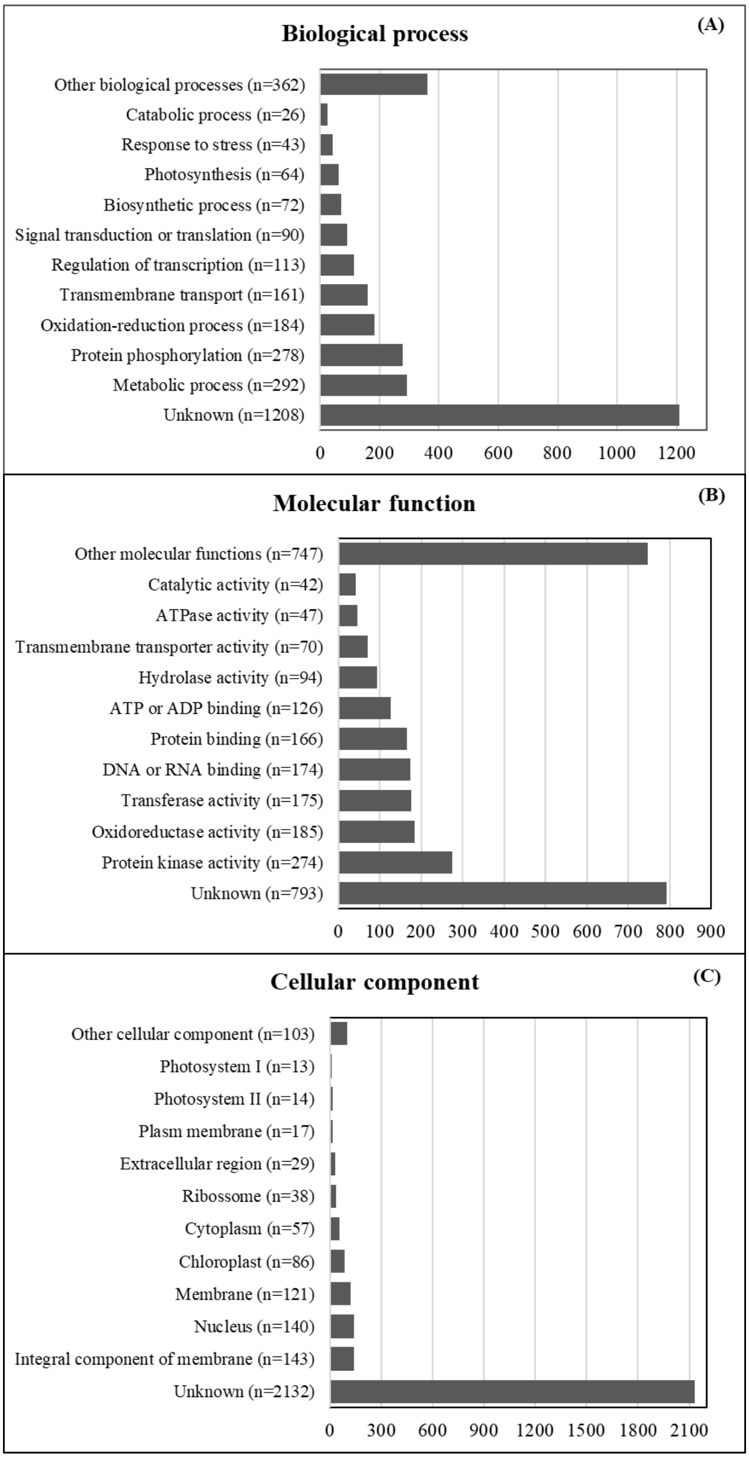


Supplementary Figure 1. Length distribution of transcripts assembled from RNA-Seq libraries of glyphosate-resistant and -sensitive biotypes of *Lolium multiflorum.*



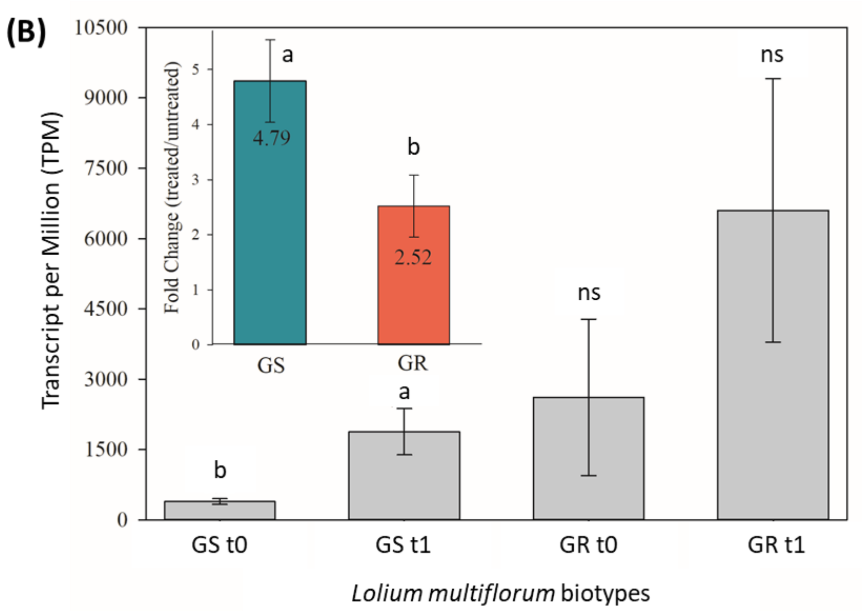
Supplementary Figure 2. Sequence comparison to other plants (hit ≥ 1%) from the distribution of BLASTx hits (e-value < 1e−10) against the non-redundant protein database of the National Center for Biotechnology Information (NCBI).



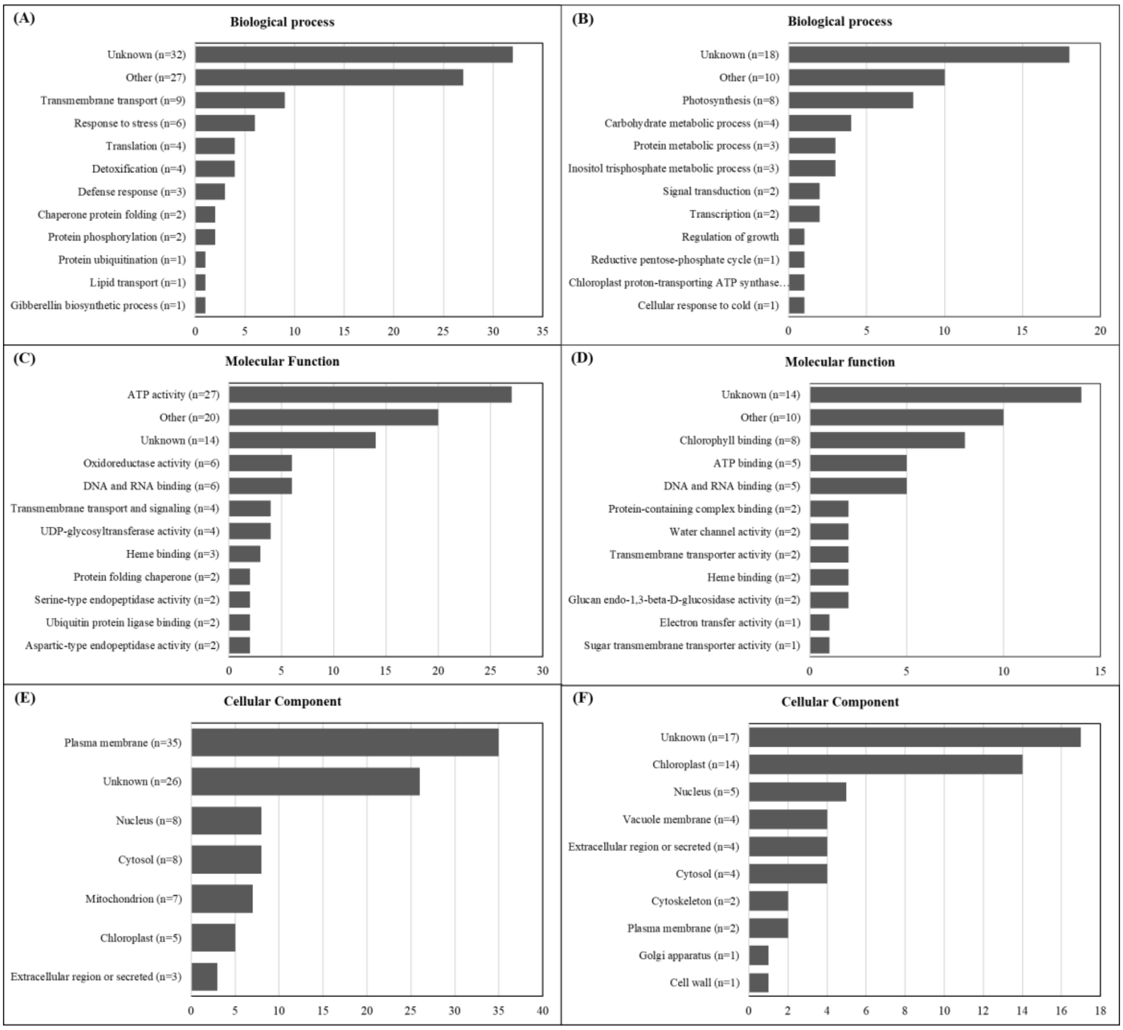
Supplementary Figure 3. Top twelve Gene Ontology (GO) terms identified in the *Lolium multiflorum* transcriptome assembly summarized in three main categories: (A) biological process, (B) molecular function, and (C) cellular component.

(A)

Amino acid position	**100**	**101**	**102**	**103**	**105**	**106**	**107**
EPSPS sequence	GCT	GGA	ACT	GCG	CGG	CCA	TTG
Translation ORF/CDS	A	G	T	A	R	P	L
Arabidopsis thaliana[1]	A	G	T	A	R	P	L
Lolium multiflorum[2]	A	G	T	A	R	P	L
Zea mays[3]	A	G	T	A	R	P	L
L. multiflorum GS	A	G	T	A	R	P	L
L. multiflorum GR	A	G	T	A	R	P	L



Supplementary Figure 4. Partial sequence alignment of the EPSPS transcripts and amino acid sequence assembled of transcriptomes from glyphosate-resistant (GR) and -sensitive (GS) biotypes compared to [1]EPSPS sequence of the *Arabidopsis thaliana* (GenBank: CAA29828.1); [2]EPSPS sequence of *Lolium multiflorum* (GenBank: DQ153168.2); [3]EPSPS sequence of *Zea mays* (GenBank: AF349754) (**A**). The grey-colored boxed amino acids show no substitution at positions Threonine 102 and Proline 106. Transcriptome expression levels (transcript reads per million mapped reads - TPM) of the EPSPS in GR and GS biotypes in response to glyphosate treatment and differential expression ratio (Fold change) (**B**). Different letters indicate significant gene expression or ratio (fold-change) in response to glyphosate treatment in each biotype by the t-test while ns means non-significant at p≤0.05. GR: glyphosate-resistant biotype; GS: glyphosate-sensitive biotype. t0: without glyphosate treatment; t1: with glyphosate treatment.



Supplementary Figure 5. Summary of top twelve gene ontology (GO) terms identified as up- (A, C, E, n=92) and down-regulated (B, D, F, n=54) genes (DEGs) in glyphosate-resistant (GR) biotype of *L. multiflorum* transcriptome in response to glyphosate treatment. Annotated sequences were classified into the biological process, molecular function, and cellular component.

Supplementary Table 1. The gene expression results of qRT-PCR (relative gene expression) and RNA-Seq (fold-change) in response to glyphosate treatment in glyphosate-resistant (GR) and -sensitive (GS) biotypes of *Lolium multiflorum* used to validate the transcriptome dataset.

Contig ID	**Uniprot ID**	**Functional Annotation**	**Putative Localization**	**Fold-Change[1,3] (qRT-PCR)**			**Fold-Change[2,3] (RNA-Seq)**		
				GR	**GS**	**Ratio GR/GS**	**GR**	**GS**	**Ratio GR/GS**
DN139722_c2_g6	**A0A3B6R885**	**EPSPs**	Chloroplast	21.9	47.8	0.46^{ns}	2.5	4.8	0.53^{ns}
DN124328_c1_g1	A0A0C5AYW1	Cinnamoyl-CoA reductase	Unknown	6.7	6.5	1.02^{ns}	30.2	23.8	1.3^{ns}
DN141928_c1_g1	R7W5T0	ABC transporter family member 2	Membrane	2.2	4.01	0.54^{ns}	1.8	3.5	0.52^{ns}
DN135064_c0_g4	M8BDG0	Cytochrome P450 78A3	Unknown	0.12	0.03	4.0*	0.034	0.006	5.5*
DN106797_c0_g1	Q25C92	Glycine-rich RNA-binding protein	Unknown	0.19	0.14	1.4^{ns}	0.15	0.11	1.4^{ns}
DN101887_c3_g1	A0A1D5UUS0	Rubisco	Chloroplast	0.26	0.29	0.9^{ns}	0.09	0.10	0.91^{ns}
DN125475_c1_g1	M8A3A2	Glutathione S-transferase GSTU6	Unknown	50.3	54.2	0.9^{ns}	18.7	17.5	1.1^{ns}
DN134465_c0_g2	M8CQB9	Heat stress transcription factor	Nucleus	6.1	8.5	0.7^{ns}	2.1	4.2	0.5^{ns}
DN110372_c0_g1	A0A287F0A7	Auxin-responsive protein	Nucleus	0.47	0.22	2.1*	0.13	0.10	1.3^{ns}
DN124884_c0_g1	Q8L686	Adenosine diphosphate glucose	Unknown	0.17	0.04	4.2*	0.05	0.008	6.2*
DN140736_c3_g2	A0A1D8MIX5	ABC transporter family member 4	Membrane	42.7	6.5	6.6*	28	3.7	7.5*
DN116297_c0_g3	A0A287E7I0	Protein detoxification	Membrane	275.5	163.6	1.7*	78.9	82.1	1^{ns}
DN139321_c2_g2	A0A1E5UIT3	Histone-lysine N-methyltransferase	Unknown	141.5	122.6	1.1^{ns}	75.5	65.4	1.1^{ns}

[1] Relative expression compared to the average of two internal control genes – 18s and eEF1As. [2] The transcriptional fold change of transcript per million (TPM). 3 Data for the GR and GS biotypes are expressed as the ratio from glyphosate treated to untreated individuals. * means significant and ^{ns} non-significant by F test ($p \leq 0.05$). The genes were randomly selected from the gene list to provide a range of gene expression, except for EPSPs. Two biotypes originated from the same geographical region (São Valentin, RS, Brazil) were used, one GR and another GS.

Supplementary Table 2. Candidate reference genes for normalization of qRT-PCR in *Lolium multiflorum* glyphosate-resistant and -sensitive biotypes in response to glyphosate treatment.

Gene	Primer Sequences[1]	Reference
18s	F- AACACTTCACCGGACCATTCA R- CGTCCCTGCCCTTTGTACAC	Zhang and Hu (2007)
GAPDH	F-AACTGTTCATGCCATCACTGCCAC R- AGGACATACCAGTGAGCTTGCCAT	Wan et al. (2010)
TUA5	F- GGCTTGTGTCTCAGGTTATCTCATC R- CATGGAGGATGGCTCGAAGG	Clarke and Rahman (2005)
eEF1As	F- CCGTTTTGTCGAGTTTGGT R- AGCAACTGTAACCGAACATAGC	Lee et al. (2010)

[1] Forward (F) and Reverse (R) primers.

Supplementary Table 3. The primers for 13 genes used for qRT-PCR analysis in *Lolium multiflorum* glyphosate-resistant and -sensitive biotypes in response to glyphosate treatment.

Gene Name	Sequence Primer
EPSPs	F- GCCGAGGAAACAATCAACAT R- GCAGGTTTCCGATTGAGAAG
Cinnamoyl-CoA reductase	F- GGTCGGCCAAGACATACG R- GGGTACTCCGGGAAGAGC
ABC transporter family member 2	F- GGGAAGCACAACACTGAAATC R- CAGGTTGTCTTAGACGGTTGC
Cytochrome P450 78A3	F- TGCATGAGTTCGAGTGGATG R- CCAAAACGGGACACTACACA
Glycine-rich RNA-binding protein	F- CGCTCTTCATCGACTCGGA R- GCAATGGCGGAAGAGTACC
Rubisco	F- CGGTGGCAGGTAGGAAAGG R- CAGGGCCTCAAGTCCACC
Glutathione S-transferase GSTU6	F- GGAGGACTCACAAACAGGCT R- GGAGGAGACGATGTGAAGCT
Heat stress transcription factor	F- GCTCAACGGGATCAGAGGT R- GGCGATGAACATGTCCCATG
Auxin-responsive protein	F- GCTCAACGGGATCAGAGGT R- GGCGATGAACATGTCCCATG
Adenosine diphosphate glucose	F- GCCGTGGTAGTAGAAGTCCC R- GCTAGCACACACAGAGTAGC
ABC transporter family member 4	F- GCTTGTTCGGCGCTTGTC R- CGGCTATCTTCACCTCTTGC
Protein detoxification	F- CGTTCTACTTGGCAGGCATC R- GGCTACCACAAACTATGCCG
Histone-lysine N-methyltransferase	F- GCCCAGGAATACTATGATGAGC R- TGTTAAGACTGTGTTGCGCG

SUPPLEMENARY SEQUENCES

ABC transporter A family member 7 - ABCA7 - TRINITY_DN140736_c3_g2; len=359

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GTTGCTAAGGGAGCACAAGCATTCAATGAAAACGTGTAACTAGTACTATCCGATTCGTAGGTGATTAT
TCTTTTCCTTTGTGAAGAAAT

Cell wall integrity and stress response - WSC2 - TRINITY_DN134601_c1_g3; len=420
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GACTCAACAGGGCTGCCGGCGGCGCCGAAGAAGAGGACCTCCTCACGGCACAGGAGAAGAAGCAGC
TCAGGAACGACAAGGACAAGCAGCGGACCAAGGACATCAACAAGGATCGACGGAATAAGAACACA
CAATATTGATGACTATGGGCGCTCAATTATCTGTTCCTTCAGCTTTCAGAGAGATTGTAATTTCCTTTAT
TTGTACTCAATTGTAGTGTTTGTTGAGATGGATTCCATGTTCATCTTTCTATATGAATGCATGTTTTGTTT
GTTTAAAAAAAAAA

COBRA-like protein 7 – COBL7 – TRINITY_DN140896_c3_g3; len=218
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TGGTCCTGTTGGTGTCCGGCGGCATCTTGAAGACCTGGATTTGGAAGGCGGATTTGGACTGGGAGGCG
TCCATGGACTTGGGCAGGATGGTGCCGTTGCGGCAGCAGTGGTTGATTCTCCCCATTTCGGTGTCGTTG
TAGCGGGAGAGCGG

Fe2OG dioxygenase domain-containing protein - TRINITY_DN138383_c1_g1; len=674
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CTGCTTCTCTTGGTTCTGAAGAAGGCGCCCCAATTGTATGCCCTGTACCTGGGCGGG

Gibberellin 20 oxidase 2 - GA20OX2 - TRINITY_DN112601_c0_g2; len=350
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CCTGCCTGGATTCAGAGAGGCACTGGAGGAGTACGCGAAGGCGATGGAGGAGCTGGCCTTGAAGCTG
ATGGAGCTGATCGCGCGCAGCCTGGGGCTGAGACCGGACCGGCTGAAGGGCTTCTTCAAGGACGACC
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CGCCACAAGGACGC

Glucan endo-1,3-beta-glucosidase - GLC1 - TRINITY_DN137545_c0_g2; len=230
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Glycosyltransferase UDP UGT13248 - TRINITY_DN130084_c1_g4; len=211
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Glycosyltransferase UDP 79 UGT79 - TRINITY_DN125138_c7_g3; len=442

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Glycosyltransferase - TRINITY_DN123097_c0_g1; len=623
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Subtilisin-chymotrypsin inhibitor - WSCI - TRINITY_DN111587_c0_g1; len=641
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Sulfate transporter 1.2 - TRINITY_DN135754_c1_g1; len=848
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Ubiquitin – TRINITY_DN120520_c4_g4; len=237
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ACCCTCGCCGACTACAACGTCCTCAAGCAGTCCACG

Ubiquitin-60S ribosomal protein L40-2 Ub-CEP52-2 – TRINITY_DN123915_c1_g2; len=251
AGCAGCTGGAGGACGGGCGCACCCTGGCCGACTACAACATCCAGAAGGAGTCGACGCTGCACCTCGT
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CTGCCGCAAGAAGAAGTGCGGCCACACCAACCAGCTCAGGCCCAAGAAG

Uncharacterized protein - TRINITY_DN141149_c4_g5; len=217
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CGAGCTCGACGT

DAO domain-containing protein - TRINITY_DN134059_c0_g2; len=398
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GATCGGCCGGTTCAAGGGCAACCCGATGGGGAATGCCATGAGTTTCTGAGATGAAATTATGAAACTG
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Glycine-rich cell wall structural protein - GRP - TRINITY_DN119890_c0_g2; len=307
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ACTAACTTGCATCTATGGATACATTTTTGTTCATGCAGAGGCTAAGAAGGAAGAGGCCAAGGCTGGCG
TGCAGGGCTACCACGGCGGCGGCGGAGGCTACTA

RNA polymerase sigma 10ator – TRINITY_DN137034_c0_g1; len=375
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GTCCAGGGCTACTGTTACACAAACCACATATGAACCAGATTTTGCACTTGCTTGGAATGTTACCGAAT
CTGGTCCCATGACTAGTCCTCTCGAGGAGCAAAGCGATGATCAACTGGTCATTCGGAGCGGGAGAAG
CCAGGAGAGGAAACTGAAGAGAATTAGAGCGTCCGAG

Senescence-associated protein - DIN1 - TRINITY_DN136212_c1_g1; len=239
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GACACTTGCTCTACAAAATGCGAGTTCTTCGACATCCCTGAGCCCGTGCTGTACATGTAAGGGATGTTC
ACGGCTCCTGCGGGGTGCCCGGCGCCGAACTCACT

Transcription factor - HBP-1b(c1) - TRINITY_DN134440_c0_g3; len=1145
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GGGGTCTGCCACATTCCAGACAGCACATGGAAAACATCTGCTTTGGCTGCTACACCCTTGAGCCTGAA
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ACTGTTAAGCAGATAACCATTAAACCAATATGCTTTGGTATAAGCTGAT

Uncharacterized protein – TRAES_3BF019300240CFD – TRINITY_DN124989_c0_g4; len=209
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CCTTGAAGGCGCAGTGATGAAGGAAGAAGCGGCCGGCAAGAAAGCCGGAGGAGCTGAGGTGATGCC
TTGGGGGATCGAGAAGTGGTGGCGGACGCACGTCACGGTGCCGGAGGTCCCCAAGTACGCGGAGAA
GGCGCCTAA

Vacuolar protein sorting-associated - VPS60-1 - TRINITY_DN127114_c0_g3; len=590
AAGCACAAGCGCATGTACGAGGATCAGCGCAACATGCTCTACAACCAGACCTACAACCTCGACCAGG
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GGAGCTTAAGGGGATGATGAAAACCGTCAAGATCGAGGATATCGATAATATGCAAGATGAAATGACG
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