M1 GENIOMHE 2024/25: Project

Structural Genomics of *Triticum aestivum*

Group members:

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Introduction

Introduction on the species, structural genomics and goal of this project, then discuss potential issues that might arise due to complexity. Proceed by summarizing the desired workflow highlighting the criteria demanded by the instructor to be fulfilled

Triticum aevistum (commonly known as wheat), is a complex eukaryotic organism belonging to kingdom Plantae, phylum Angiosperms, class Monocots, order Poales, family Poaceae, genus Triticum. This plant has been considered as one of the most important crops in the world, providing a staple food source for billions of people as it is mainly used to make bread.

Even though it is somehow considered a model organism in plant biology, it has a complex genome structure that makes it difficult to study. It is a hexaploid species with a large genome, consisting of 7n chromosomes. This polyploidy is in fact derived from the hybridization of three different species due to an evolutionary event that occurred around 8,500–9,000 years ago. It comes from a

tetraploid species having BBAA chromosomes and a diploid species having DD chromosomes. The tetraploid species is believed to be a free-threshing species and can be thought to be *Triticum monococcum* or *Triticum durum*, whereas the diploid species is *Aegilops tauschii*. Ending up with a hexaploid species with BBAADD chromosomes.[1]

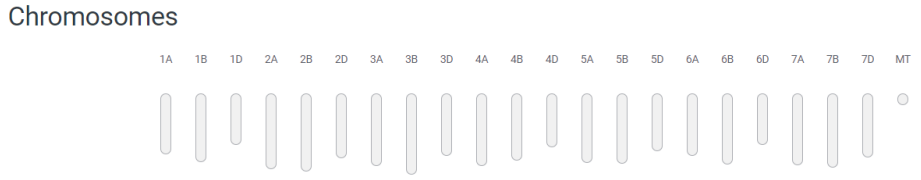


Figure 1: T. aestivum set of chromosomes from RefSeq

The goal of this project is to annotate a specific region of the genome of *Triticum aestivum* (wheat), mainly structurally annotate, using bioinformatics tools. The region of interest is a 14,001 bp sequence (`region8`), which we will analyze to predict genes, transposable elements, and other features. This task is considerably a hard one taking into consideration this complicated genome structure from polyploidy and the richness of repetitive elements, as well as its large size.
In this project, we will start of by a minor exploration fo our region then we'll perform gene prediction using a variety of tools then analyze and validate these results. We will also look for transposable elements in the region and perform a final annotation of the region to conclude with this report. We have used online servers, databases, api calls, unix tools, visualization software, python & bash scripting to perform the analysis. Supplementary results, data, code, figures and documentation can be found on the github repository of this project: github.com/raysas/wheat-seq-annotation.

Exploration

Sequence properties

Checking GC content in this region to have an idea about potential gene desnitites. For that we run the script:

```
$ python src/GCcontent.py data/region8.fasta
0.48
```

The GC content of the DNA sequence is 48%.

We proceed to see the length of the sequence:

```
$ expr $(tail -n +2 data/region8.fasta | wc -c) - $(tail -n +2 data/region8.fasta | wc -l)
14001
```

[1] Levy, Avraham A., and Moshe Feldman. "Evolution and origin of bread wheat." The Plant Cell 34.7 (2022): 2549-2567.

region8 is 14,001 bases long.

Region localization

Want to localize this region by mapping agaisnt the reference sequence of *Triticum aestivum* (available on RefSeq at GCF_018294505.1), which consists of $7n$ chromosomes[2]. After retrieving the reference sequence, we perfomed mapping through Burrows-Wheeler Aligner MEM (bwa-mem) algorithm, and due to large genome size, we did this step on Galaxy because of the large computation time and memory required.

```
$ bwa-mem2 mem -t 4 data/sequences/reference/GCF_018294505.1_genomic.fna \
    data/region8.fasta > data/sequences/alignment/region8.sam
$ samtools sort data/sequences/alignment/region8.sam \
    > data/sequences/alignment/region8_aln.bam
$ bedtools bamtobed -i data/sequences/alignment/region8_aln.bam \
    > data/sequences/alignment/region8_aln.bed
```

We chose this mapper because it's perfect for medium length reads ranging between 100bp and megabases, in our case it's a 14kb sequence

Now we have in the output a `.bam` file and a `.bed` file. From the `.bam` file we can get the following information when running the following command:

```
$ samtools view -c -F 4 data/sequences/alignment/region8_aln.bam
```

[2]

Chromosome	GenBank	RefSeq	Size (bp)	GC content (%)	Unlocalized count	Action
1A	CM031178.1	NC_057794.1	598,660,471	46	0	
1B	CM031179.1	NC_057795.1	700,547,350	46	0	
1D	CM031180.1	NC_057796.1	498,638,509	46.5	0	
2A	CM031181.1	NC_057797.1	787,782,082	46	0	
2B	CM031182.1	NC_057798.1	812,755,788	46	0	
2D	CM031183.1	NC_057799.1	656,544,405	46.5	0	
3A	CM031184.1	NC_057800.1	754,128,162	46	0	
3B	CM031185.1	NC_057801.1	851,934,019	46	0	
3D	CM031186.1	NC_057802.1	619,618,552	46.5	0	
4A	CM031187.1	NC_057803.1	754,227,511	46	0	
4B	CM031188.1	NC_057804.1	673,810,255	46.5	0	
4D	CM031189.1	NC_057805.1	518,332,611	46.5	0	
5A	CM031190.1	NC_057806.1	713,360,525	46	0	
5B	CM031191.1	NC_057807.1	714,697,677	46	0	
5D	CM031192.1	NC_057808.1	569,951,140	46.5	0	
6A	CM031193.1	NC_057809.1	622,669,697	46	0	
6B	CM031194.1	NC_057810.1	731,188,232	46.5	0	
6D	CM031195.1	NC_057811.1	495,380,293	46.5	0	
7A	CM031196.1	NC_057812.1	744,491,536	46	0	
7B	CM031197.1	NC_057813.1	764,072,961	46	0	
7D	CM031198.1	NC_057814.1	642,921,167	46.5	0	
MT	EU534409.1	NC_036024.1	452,526	44.5	0	

```
region8 16      NC_057805.1     497158671       60      9565M1I4435M    *       0       0
<sequence>   *  NM:i:5   MD:Z:9565A1651C131G821T1828     AS:i:13973      XS:i:2788
```

From the `.bam` output we can see[3]:

- The CIGAR string `9565M1I4435M`, means that the read is 9565 bases long, then there is an insertion of 1 base, and then 4435 more bases.
- The `NM:i:5` field indicates that there are 5 mismatches in the alignment.
- The `MD:Z:9565A1651C131G821T1828` field indicates the mismatches in the alignment.

If we further proceed conversion onto a `.bed` file, we get the following info:

```
NC_057805.1 497158670   497172670   region8 60  -
```

This means that the region8 is:

- located on the chromosome `NC_057805.1`
- position starting from `497158670` and ending at `497172670`
- on the negative strand.

Reflection: our sequence is of length 14469, and the read is 9565+1+4435=14001, which means that the alignment is EXACTLY the same length as the sequence, and the 5 mismatches are not significant relative to the number of bases. We can thus infer that region8 is well mapped to the reference genome on the negative strand of chromosome `NC_057805.1` starting at position `497158670` and ending at `497172670`. And according to the table in [4] retrieved from RefSeq, this

[3] Li, Heng, et al. "The sequence alignment/map format and SAMtools." bioinformatics 25.16 (2009): 2078-2079.

[4]

Chromosome	GenBank	RefSeq	Size (bp)	GC content (%)	Unlocalized count	Action
1A	CM031178.1	NC_057794.1	598,660,471	46	0	
1B	CM031179.1	NC_057795.1	700,547,350	46	0	
1D	CM031180.1	NC_057796.1	498,638,509	46.5	0	
2A	CM031181.1	NC_057797.1	787,782,082	46	0	
2B	CM031182.1	NC_057798.1	812,755,788	46	0	
2D	CM031183.1	NC_057799.1	656,544,405	46.5	0	
3A	CM031184.1	NC_057800.1	754,128,162	46	0	
3B	CM031185.1	NC_057801.1	851,934,019	46	0	
3D	CM031186.1	NC_057802.1	619,618,552	46.5	0	
4A	CM031187.1	NC_057803.1	754,227,511	46	0	
4B	CM031188.1	NC_057804.1	673,810,255	46.5	0	
4D	CM031189.1	NC_057805.1	518,332,611	46.5	0	
5A	CM031190.1	NC_057806.1	713,360,525	46	0	
5B	CM031191.1	NC_057807.1	714,697,677	46	0	
5D	CM031192.1	NC_057808.1	569,951,140	46.5	0	
6A	CM031193.1	NC_057809.1	622,669,697	46	0	
6B	CM031194.1	NC_057810.1	731,188,232	46.5	0	
6D	CM031195.1	NC_057811.1	495,380,293	46.5	0	
7A	CM031196.1	NC_057812.1	744,491,536	46	0	
7B	CM031197.1	NC_057813.1	764,072,961	46	0	
7D	CM031198.1	NC_057814.1	642,921,167	46.5	0	

chromosome is the 4D chromosome of *Triticum aestivum*.

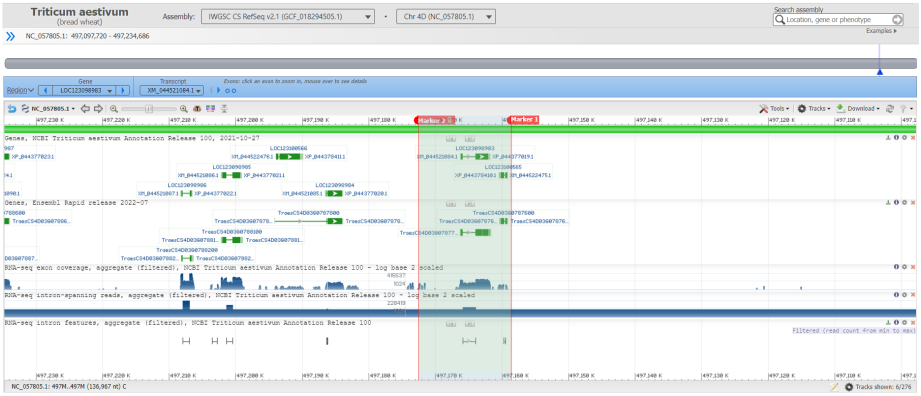


Figure 2: Start and End positions of the alignment on the reference genome - RefSeq Genome Browser (marker 1: start position; marker 2: end position)

We can visualize the `.bed` file in Ensembl Plants, IWGSC assembly converter

Moreover, this region has a GC content of 48% (as reported earlier), which is ~2% higher than the average GC content of the whole 4D chromosome (46.5%). This might indicate a high gene density in this region, as genes are known to have a higher GC content than the rest of the genome.

Gene Prediction

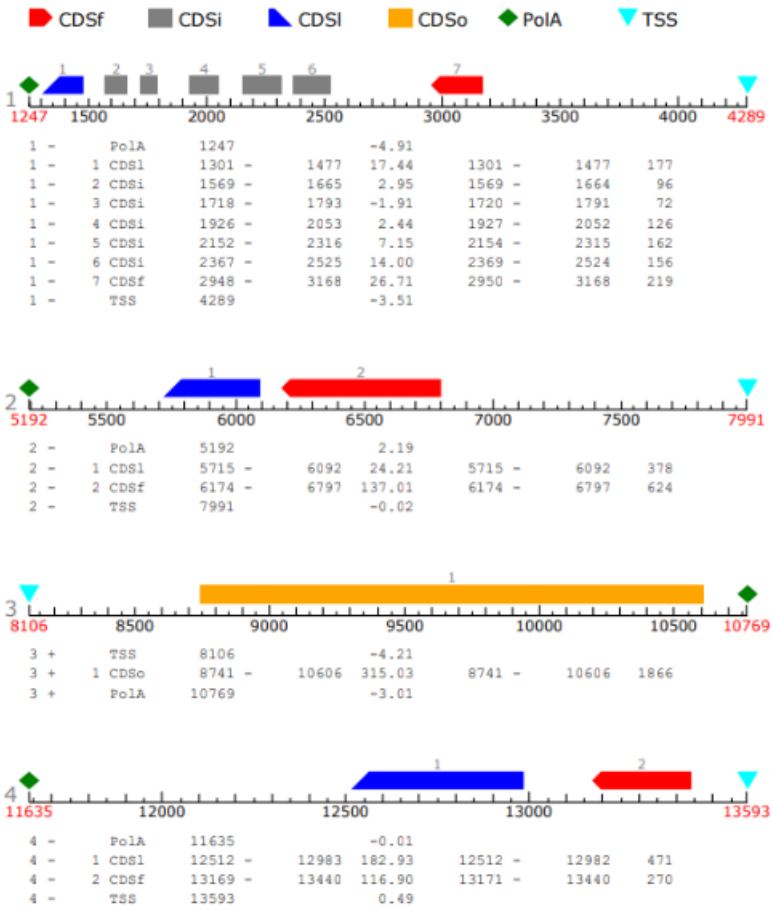
Tools

FGENESH

We used the FGENESH site, providing only the name of the organism, Triticum aestivum (wheat), and the DNA sequence. The default settings used a gene prediction model specifically trained for Triticum aestivum, which allowed the software to identify potential genes, exons, and other features such as transcription start sites (TSS) and polyadenylation sites (PolA). The output includes the positions of coding sequences : The parts of exons encoding proteins, TSS (Transcription Start Site): Where transcription begins, PolA (Polyadenylation Site): Where mRNA processing ends.
The FGENESH analysis of a 14,001 bp Triticum genomic DNA sequence predicted four genes, with one on the positive strand and three on the negative strand. In total, 12 exons were identified, with one on the positive strand and 11 on the negative strand.

MT	EU534409.1	NC_036024.1	452,526	44.5	0

FGENESH 2.6 Prediction of potential genes in Triticum genomic DNA
Seq name: test sequence
Length of sequence: 14001
Number of predicted genes 4: in +chain 1, in -chain 3.
Number of predicted exons 12: in +chain 1, in -chain 11.
Positions of predicted genes and exons: Variant 1 from 1, Score:813.705811



1	-		PolA	1247		-4.91			
1	-	1	CDSl	1301	-	1477	17.44	1301 -	1477	177
1	-	2	CDSi	1569	-	1665	2.95	1569 -	1664	96
1	-	3	CDSi	1718	-	1793	-1.91	1720 -	1791	72
1	-	4	CDSi	1926	-	2053	2.44	1927 -	2052	126
1	-	5	CDSi	2152	-	2316	7.15	2154 -	2315	162
1	-	6	CDSi	2367	-	2525	14.00	2369 -	2524	156
1	-	7	CDSf	2948	-	3168	26.71	2950 -	3168	219
1	-		TSS	4289		-3.51			

2	-		PolA	5192		2.19			
2	-	1	CDSl	5715	-	6092	24.21	5715 -	6092	378
2	-	2	CDSf	6174	-	6797	137.01	6174 -	6797	624
2	-		TSS	7991		-0.02			

3	+		TSS	8106		-4.21			
3	+	1	CDSo	8741	-	10606	315.03	8741 -	10606	1866
3	+		PolA	10769		-3.01			

4	-		PolA	11635		-0.01			
4	-	1	CDSl	12512	-	12983	182.93	12512 -	12982	471
4	-	2	CDSf	13169	-	13440	116.90	13171 -	13440	270
4	-		TSS	13593		0.49			

Figure 3: FGENESH results

Gene 1, located on the negative strand, extends from position 1301 to 3168 and contains 7 exons, starting with the first coding exon (CDSl) at 1301–1477 and ending with the last exon (CDSf) at 2948–3168. It also includes 5 intermediate coding exons at positions 1569–1665, 1718–1783, 1926–2053, 2152–2316, and 2367–2525. The transcription start site (TSS) is identified at position 4289, while the polyadenylation site (PolA) is located at 1247. This gene translates into a protein of 340 amino acids.
Gene 2, also on the negative strand, extends from 5715 to 6797 and contains 2 exons, with the first coding exon (CDSl) at 5715–6092 and the last exon (CDSf) at 6174–6797, producing a protein of 333 amino acids. The Transcription start site (TSS) is identified at position 7991, while polyadenylation site (PolA) is located at 5192.
Gene 3, on the positive strand, is a single-exon gene (CDSo) located between 8741 and 10606, encoding a protein of 621 amino acids. The Transcription start site (TSS) is identified at position 8106, while polyadenylation site (PolA) is located at 10769.
Gene 4, on the negative strand, spans 12512–13440 with 2 exons; the first coding exon (CDSl) is at 12512–12983, and the final exon (CDSf) is at 13169–13440, translating into a protein of 247 amino acids. Transcription start site (TSS) is identified at position 13593, while polyadenylation site (PolA) is located at 11635.
The gene features, including exon positions and their strand orientation, suggest diverse transcriptional structures, with detailed sequences provided for both mRNA and proteins.

GENEID

Gene 1 is located on the forward strand (+) and consists of two exons, with the first exon positioned from 6532 to 6762 and the terminal exon from 7754 to 7759. Gene 2 is also on the forward strand (+) and is a single-exon gene, extending from 10160 to 10606. In contrast, Gene 3 is on the reverse strand (-) and has two exons, with the terminal exon located between 12512 and 12983, and the first exon from 13169 to 13434. The annotation reflects the strand orientation, with Gene 1 and Gene 2 being forward-strand genes, while Gene 3 is on the reverse strand, where exons are annotated in reverse order, starting from the terminal exon

AUGUSTUS

The AUGUSTUS gene prediction tool (version 3.3.3) analyzed a 14,001 bp sequence using the wheat parameter set and identified two genes, one on the forward strand and one on the reverse strand. Gene 1 on the forward strand, extends from 6226 to 10861 and contains two exons separated by an intron. The start codon is located in exon 1 (6532–6534), while the stop codon is in exon 2 (10604–10606). The coding sequence (CDS) includes two segments: 6532–6762 and 8714–10606. Gene 2, on the reverse strand, spans positions

```
# start gene g1
unnamed-1       AUGUSTUS        gene    6226    10861   0.03    +       .
unnamed-1       AUGUSTUS        transcript      6226    10861   0.03    +
unnamed-1       AUGUSTUS        tss     6226    6226    .       +       .
unnamed-1       AUGUSTUS        exon    6226    6762    .       +       .
unnamed-1       AUGUSTUS        start_codon     6532    6534    .       +
unnamed-1       AUGUSTUS        initial 6532    6762    0.94    +       0
unnamed-1       AUGUSTUS        terminal        8714    10606   0.93    +
unnamed-1       AUGUSTUS        intron  6763    8713    0.89    +       .
unnamed-1       AUGUSTUS        CDS     6532    6762    0.94    +       0
unnamed-1       AUGUSTUS        CDS     8714    10606   0.93    +       0
unnamed-1       AUGUSTUS        exon    8714    10861   .       +       .
unnamed-1       AUGUSTUS        stop_codon      10604   10606   .       +
unnamed-1       AUGUSTUS        tts     10861   10861   .       +       .

# start gene g2
unnamed-1       AUGUSTUS        gene    12415   13535   0.06    -       .
unnamed-1       AUGUSTUS        transcript      12415   13535   0.06    -
unnamed-1       AUGUSTUS        tts     12415   12415   .       -       .
unnamed-1       AUGUSTUS        exon    12415   12983   .       -       .
unnamed-1       AUGUSTUS        stop_codon      12512   12514   .       -
unnamed-1       AUGUSTUS        terminal        12512   12983   1       -
unnamed-1       AUGUSTUS        initial 13169   13434   0.74    -       0
unnamed-1       AUGUSTUS        intron  12984   13168   1       -       .
unnamed-1       AUGUSTUS        CDS     12512   12983   1       -       1
unnamed-1       AUGUSTUS        CDS     13169   13434   0.74    -       0
unnamed-1       AUGUSTUS        exon    13169   13535   .       -       .
unnamed-1       AUGUSTUS        start_codon     13432   13434   .       -
unnamed-1       AUGUSTUS        tss     13535   13535   .       -       .
```

Figure 4: AUGUSTUS results

12415–13535 and also contains two exons with an intron between them. The stop codon is in exon 1 (12512–12514), and the start codon is in exon 2 (13432–13434). The CDS includes two regions: 12512–12983 and 13169–13434. Both genes encode functional proteins. This detailed output highlights exon-intron boundaries, coding regions, and predicted protein sequences, which are valuable for downstream analyses like functional annotation and experimental validation.

DNA Subway AUGUSTUS

Seqid	Source	Type	Length	Start	End	Score	Strand	Phase	Attributes
wheat_53611	AUGUSTUS	gene	4075	6532	10606	0.87	+	.	Name=AUGUSTUS001;ID=g1
wheat_53611	AUGUSTUS	mRNA	4075	6532	10606	0.87	+	.	ID=g1.t1;Parent=g1
wheat_53611	AUGUSTUS	CDS	231	6532	6762	1	+	0	Parent=g1.t1
wheat_53611	AUGUSTUS	exon	231	6532	6762	1	+	0	Parent=g1.t1
wheat_53611	AUGUSTUS	CDS	25	8714	8738	0.87	+	0	Parent=g1.t1
wheat_53611	AUGUSTUS	exon	25	8714	8738	0.87	+	0	Parent=g1.t1
wheat_53611	AUGUSTUS	CDS	97	8824	8920	0.87	+	2	Parent=g1.t1
wheat_53611	AUGUSTUS	exon	97	8824	8920	0.87	+	2	Parent=g1.t1
wheat_53611	AUGUSTUS	CDS	1615	8992	10606	0.87	+	1	Parent=g1.t1
wheat_53611	AUGUSTUS	exon	1615	8992	10606	0.87	+	1	Parent=g1.t1
wheat_53611	AUGUSTUS	gene	929	12512	13440	0.54	-	.	Name=AUGUSTUS002;ID=g2
wheat_53611	AUGUSTUS	mRNA	929	12512	13440	0.54	-	.	ID=g2.t1;Parent=g2
wheat_53611	AUGUSTUS	CDS	472	12512	12983	0.88	-	1	Parent=g2.t1
wheat_53611	AUGUSTUS	exon	472	12512	12983	0.88	-	1	Parent=g2.t1
wheat_53611	AUGUSTUS	CDS	272	13169	13440	0.54	-	0	Parent=g2.t1
wheat_53611	AUGUSTUS	exon	272	13169	13440	0.54	-	0	Parent=g2.t1

Figure 5: DNA subway AUGUSTUS results

The AUGUSTUS tool identified two genes, g1 and g2, in the wheat sequence wheat_53611. Gene 1, located on the forward strand, spans positions 6532–10606 with a length of 4075 bp and a high prediction score of 0.87. It consists of four exons, with CDS regions ranging from 6532–6762, 8714–8738, 8824–8920, and 8992–10606. Gene 2, located on the reverse strand, spans positions 12512–13440 with a length of 929 bp and a prediction score of 0.54. It contains two exons, with CDS regions spanning 12512–12983 and 13169–13440. These predictions highlight the structural details of both genes, including exon-intron boundaries and coding sequences, which are critical for downstream analyses

such as functional annotation and protein prediction.

DNA Subway FGENESH

Seqid	Source	Type	Length	Start	End	Score	Strand	Phase	Attributes
wheat_53611	FGenesH	gene	4075	6532	10606	.	+	.	Name=FGENESH001;ID=gf001
wheat_53611	FGenesH	mRNA	4075	6532	10606	.	+	.	ID=gf001.1;Parent=gf001
wheat_53611	FGenesH	exon	231	6532	6762	21.81	+	.	Parent=gf001.1
wheat_53611	FGenesH	CDS	231	6532	6762	21.81	+	.	Parent=gf001.1
wheat_53611	FGenesH	exon	1893	8714	10606	120.25	+	.	Parent=gf001.1
wheat_53611	FGenesH	CDS	1893	8714	10606	120.25	+	.	Parent=gf001.1
wheat_53611	FGenesH	gene	923	12512	13434	.	-	.	Name=FGENESH002;ID=gf002
wheat_53611	FGenesH	mRNA	923	12512	13434	.	-	.	ID=gf002.1;Parent=gf002
wheat_53611	FGenesH	exon	216	12512	12727	9.77	-	.	Parent=gf002.1
wheat_53611	FGenesH	CDS	216	12512	12727	9.77	-	.	Parent=gf002.1
wheat_53611	FGenesH	exon	49	13072	13120	-7.58	-	.	Parent=gf002.1
wheat_53611	FGenesH	CDS	49	13072	13120	-7.58	-	.	Parent=gf002.1
wheat_53611	FGenesH	exon	266	13169	13434	33.00	-	.	Parent=gf002.1
wheat_53611	FGenesH	CDS	266	13169	13434	33.00	-	.	Parent=gf002.1

Figure 6: DNA Subway FGENESH

The FGENESH tool identified two genes, gf001 and gf002, in the wheat sequence wheat_53611. Gene gf001, located on the forward strand, spans positions 6532–10606 with a length of 4075 bp. It consists of two exons, the first spanning 6532–6762 (231 bp, score 21.81) and the second spanning 8714–10606 (1893 bp, score 120.25). Both exons contribute to the coding sequence (CDS). Gene gf002, located on the reverse strand, spans positions 12512–13434 with a length of 923 bp. It contains three exons: the first spans 12512–12727 (216 bp, score 9.77), the second spans 13072–13120 (49 bp, score -7.58), and the third spans 13169–13434 (266 bp, score 33.00). These detailed annotations provide insights into gene structures, exon positions, and strand orientation, making them valuable for downstream analysis and functional studies.

Gene Position	Fgenesh (exons)	DNA Subway Fgenesh (exons)	Augustus (exons)	DNA Subway Augustus (exons)	Geneid (exons)
Gene 1	1301–3168 (-) (7 exons)	6532–10606 (+) (2 exons)	6226–10861 (+) (2 exons)	6532–10606 (+) (4 exons)	6532–7759 (+) (2 exons)
Gene 2	5715–6797 (-) (2 exons)	12512–13434 (-) (3 exons)	12415–13535 (-) (2 exons)	12512–13440 (-) (2 exons)	10160–10606 (+) (1 exon)
Gene 3	8741–10606 (+) (1 exon)	-	-	-	-
Gene 4	12512–13440 (-) (2 exons)	-	-	-	12512–13434 (-) (2 exons)

	Fgenesh	DNA subway Fgenesh	Augustus	DNA subway Augustus	Geneid
gene 1 position	1301 - 3168 (-) (7exons)	6532-10606 (+) (2 exons)	6226-10861 (+) (2 exons)	6532-10606 (+) (4exons)	6532-7759(+) (2 exons)
gene 2 position	5715 - 6797 (-) (2 exons)	12512-13434(-) (3 exons)	12415-13535 (-) (2 exons)	12512-13440(-) (2 exons)	10160-10606 (+) (1exon)
gene 3 position	8741 - 10606 (+) (1exon)				12512-13434 (-) (2 exons)
gene 4 position	12512 - 13440(-) (2 exons)				

Figure 7: Table with colored labels for follow up

Common regions:

- **Gene 1** (green region) : The gene spanning from 6532 to 10606 on the forward strand (+), marked in green in the results of the DNA subway Fgenesh, is a consistent feature across multiple gene prediction tools but with some variations. Augustus and DNA subway augustus predict this gene at a slightly extended position from 6226 to 10861 with 2 exons for augustus and from 6532 to 10606 with 4 exons for DNA subway augustus. Geneid also identifies this region but divides it into two separate predictions: one from 6532 to 7759 (2 exons) and another one from 10160 to 10606 (1exon). This split in Geneid's prediction suggests a possible alternative structure or fragmentation. FGENESH, while differing in interpretation, may be representing the same gene with variation, as it predicts a single-exon gene extending from 8741 to 10606 (the green region) on the forward strand, aligning with the green region predicted in the other tools.

For the other region predicted by FGENESH that extends from 5715 to 6797 (the blue region) on the reverse strand (-) with 2 exons, it is not supported by the other tools. This region may partially overlap with predictions made by tools that focus on nearby regions but it does not appear explicitly as a standalone gene in the results of tools like AUGUSTUS or GENEID or DNA subway Fgenesh.

- **Gene 2** (yellow region) : the gene extending from 12512 to 13434 on the reverse strand (-) with 3 exons, marked in yellow in the results of the DNA subway Fgenesh, is a consistent feature across multiple gene prediction tools but with slight differences in exon count and exact positions. FGENESH predicts this region as a gene with 2 exons, extending from 12512 to 13440, aligning with the prediction of DNA subway Fgenesh. Augustus predicts this region as a gene spanning from 12415 to 13535 with 2 exons, slightly extending the boundaries compared to FGENESH. GENEID matches closely with FGENESH, predicting this gene at 12515 to 13434 with 2 exons. This consistency in identifying this region across tools indicates that it is a reliable gene prediction, with the variations in exon count and precise start-end positions reflecting the differences in each tool's algorithm.

Non common regions:

The region highlighted in *pink* (1301-3168 on the reverse strand) is a unique prediction made exclusively by FGENESH. According to FGENESh, this region has 7 exons starting from 1301 and ending at 3168. This prediction is not supported by any of the other tools used in the analysis, such as DNA Subway FGENESH, AUGUSTUS or Geneid which do not identify a gene in this specific region. The lack of agreement from other tools suggests that this region might be an artifact of the FGENESH algorithm, a false positive, or a region with characteristics that make it detectable only by FGENESH. Alternatively, it could represent a low-confidence or poorly conserved gene that is difficult for other tools to detect

$\Longleftrightarrow$ These variations between the tools highlight the need for further investigation, validation as through transcriptomic data, to confirm the existence, structure, and functionality of these predicted genes and to ensure biologically meaningful results.

Visualization

In order to visualize the features predicted by the abovementioned tools on artemis we first need to convert them to `.gff` format.

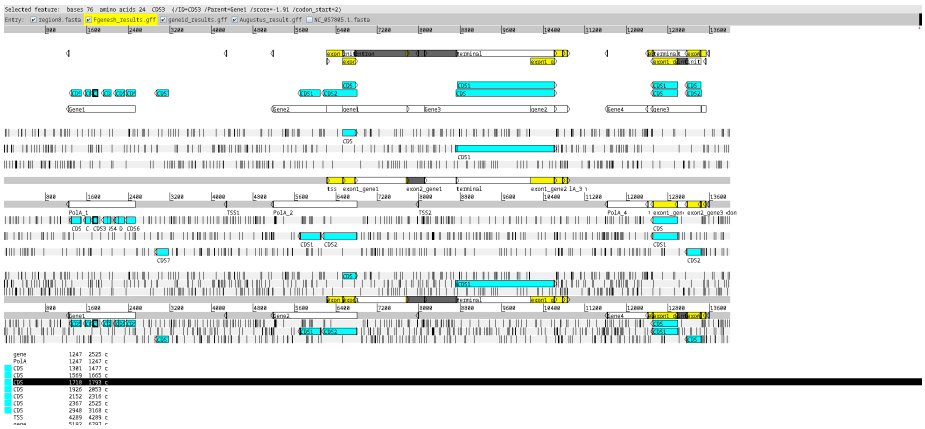


Figure 8: Predicted genes viz on Artemis

Gene Validation

BLAST

We will perform a BLAST search of the predicted genes against related species proteomes locally, using blast+ package on unix terminal.

> Blasting done through `blastp` program on the predicted genes from region8 (each tool generated .faa fasta file that is amino acid sequences, each sequence will be a query).

The proteomes were retrieved from UniProt as it is recommended to be the way

to download proteome for a whole species by an EMBL-EBI training course on UniProt[5], we will provide an api to retrieve the sequences as well to make our work replicable.

Since we're blasting against local databases built from proteomes retrieved from UniProt, the resulting hits have UniProt IDs, instead of product or gene names, thus an extra processing step was taken to annotate the results through a python script using `biopython`'s `ExPASy` and `SwissProt` modules.

> On another note, the advantages of blasting locally here on particular species is it's more specific and centered towards the species of interest, and provides a larger set of similar proteins in comparison to swissport for instance, which has a very limited number of reviewed proteins in each of the species we are interested in, which will be noted in the results.

Tritium aestivum proteome

To validate the predicted genes, we will start of by blasting against the proteome of *Triticum aestivum* available on UniProt. We retrieved the list of proteins from the supplementary material of an International Wheat Genome Sequencing Consortium (IWGSC) published in *Science*[6] aiming to provide an annotated reference sequence of the *Triticum aestivum* genome. The article is available here. We will access all the proteins sequences (including isoforms) using an api call to the UniProt database.

```
$ curl https://rest.uniprot.org/uniprotkb/stream?compressed=true&format=fasta&query=%28%28li
    > data/sequences/proteome/Triticum_aestivum_proteins.fasta.gz
$ gunzip data/sequences/proteome/Triticum_aestivum_proteins.fasta.gz
$ cat data/sequences/proteome/Triticum_aestivum_proteins.fasta | grep '>' | wc -l
130283
```

There is a total of 130,283 proteins in the file. We will now perform a BLAST search against this database to see if our predicted genes are similar to any of the known annotated proteins of *Triticum aestivum*. It'll be a blastp search, as we are looking for protein sequences that are similar to our predicted sequence (which is already translated by our tools output and saved in out repository in .faa files)

We provided the commands to make blast databases and perform the search in the `blast.sh` script.

```
./src/blast.sh
```

[5]EMBL-EBI training course on UniProt: https://www.ebi.ac.uk/training/online/courses/uniprot-exploring-protein-sequence-and-functional-info/

[6]The International Wheat Genome Sequencing Consortium (IWGSC) et al. ,Shifting the limits in wheat research and breeding using a fully annotated reference genome.Science361,eaar7191(2018).DOI:10.1126/science.aar7191

Related species

Starting from the following information:
- *Triticum monococcum* and *Triticum durum* have the A and B chromosomes
- *Aegilops tauschii* has the D chromosome

We will also look for their proteomes and perform the same blasting procedure as above.

N.B: we couldn't find *Tri. monococcum* proteome on UniProt, so we will only blast against *Tri. durum* for the common A and B chromosomes.

Triticum durum The proteome can be find on this UniProt page, 188,826 proteins, worth noting that only 2 of them are expertly reviewed - Swiss-Prot - the rest are unreviewed - TrEMBL.

For *Triticum durum*, retrieving the proteome through this api call:

```
$ curl https://rest.uniprot.org/uniprotkb/stream?compressed=true&format=fasta&query=%28%28ta
    > data/sequences/proteome/Triticum_durum_proteins.fasta.gz
$ gunzip data/sequences/proteome/Triticum_durum_proteins.fasta.gz
```

Aegilops tauschii The proteome can be find on *this UniProt page*, 214,193 proteins, only one of them is expertly reviewed.

Retrieving the proteome through this api call:

```
$ curl https://rest.uniprot.org/uniprotkb/stream?compressed=true&format=fasta&query=%28%28ta
    > data/sequences/proteome/Aegilops_tauschii_proteins.fasta.gz
$ gunzip data/sequences/proteome/Aegilops_tauschii_proteins.fasta.gz
```

Results

In this results section we are, as explained, expecting to have for each predicted gene 3 blasting results. Since we're taking into consideration 4 genes from FGENESH and 2 from AUGUSTUS and blasting against 3 species' proteomes separately (T. aestivum, A. tauschii and T. durum), we would have 6 genes to analyse with 3 resulting blast output each. *BLAST results can be found by clicking on*: this link
In the first reported blast we will analyse every single detail extensively to give an intuition of our analysis

Augustus gene 1: (*protein length: 707aa*) In the first BLAST results for AUGUSTUS against our own species's proteome *Triticum aestivum*:
We find the top 5 hits quite significant with % id higher than 98.7% and then immediately drops to 52% after these 5 matches from the proteome database, providing an e-value estimated by blast+ to be 0 which is quite significant, thus we will be considering them as top results. Looking into them, we find the 1st hit to be 100% id, found on chromosome 4D, matching all of the protein's length

(1-707 residues) against the full length of the subject from the database (also 1-707) with NO mismatches NO gaps, and this exact match gives a product: *Anaphase-promoting complex subunit 11 {ECO:0008006|Google:ProtNLM}, with gene name CFC21_063427 (as retrieved from uniprot's api)*

a snippet of the blast results and retrieved information for this hit:

- **Transcript ID:** AUGUSTUS_g1.t1
- **Protein ID:** tr|A0A3B6JR29|A0A3B6JR29_WHEAT
- **Alignment Score:** 100
- **Query Length:** 707
- **Mismatch Count:** 0
- **Gap Count:** 0
- **Query Start:** 1
- **Query End:** 707
- **Subject Start:** 1
- **Subject End:** 707
- **E-value:** 0
- **Bit Score:** 1462
- **Protein Name:** RecName: Full=Anaphase-promoting complex subunit 11 {ECO:0008006|Google:ProtNLM}
- **Chromosome:** Chromosome 4D
- **Organism:** Triticum aestivum (Wheat)
- **Organism Protein ID:** A0A3B6JR29_WHEAT
- **ORF Names:** CFC21_063427_063427 {ECO:0000313|EMBL:KAF7055962.1}
- **Keywords:** Metal-binding, Reference proteome, Zinc, Zinc-finger

All the hits follows have the same product name, same gene name and aroudn same length (707 or 708 due to inserted gap). We can notice that not all of them are on the several hits can be due to:

- duplication
- isotopes (not an expertly reviewed database liek swissprot)
- hits on the same protein sequence but different alignments patterns

Having the match 100% id to the first protein is a validation besides all the above mentioned signs from results (consistency of the matched proteins among the best hit), meaning that this gene moght be infat the CFC21_063427 gene, and the protein is the Anaphase-promoting complex subunit 11.
More interestingly it resides on the chromosome 4D, which is the chromosome we have mapped our region to, which provide a stringent evidence of our corelated work.

The 2nd blast is done on *Aegilops tauschii* proteome:

we find the 1st hit to be 100% id, matching all of the protein's length (1-707 residues) against the full length of the subject from the database (also 1-707) with

only 5 mismatches, and this exact match gives a product: *Anaphase-promoting complex subunit 11 {ECO:0008006|Google:ProtNLM} (as retrieved from uniprot's api)*

The 3 hits that follows are truncated to be around 600 residues of length (630, 629, 621 respectively) and 4 mismatches beginning at around 78-87th position of the query until the end, each corresponding to these proteins: *Anaphase-promoting complex subunit 11 (twice) and VWFA domain-containing protein {ECO:0008006|Google:ProtNLM}*.

And the last hit is strictly a small segment from the first 388 residues of the query corresponding to *RING-type domain-containing protein {ECO:0008006|Google:ProtNLM}*, which can be a suggesting that the first part of the query contains this particular domain.

Even though the annotations are quite different, 2 interesting things are worth noting: All of them are metal-binding domains containing proteins relating to zinc and zinc-fingers, as can be seen from the list of keywords extracted from uniprot from the hit id, thus showing even though there is no consensus towards the annotation there is majority agreement on the functionality and domains of this sequence product:

> N.B. proteins all show to be on the same segment (chromosome), now it's worth noting that these are not expertly annotated as found in databases like swiss port, but overall these proteins are very highly similar between each other too.

Next one with *Triticum durum* proteome, which only has A and B chromosome (and in our case we have a D chromosome) but also showed 3 significant results belonging to the same protein (CFC21_063427 gene), the 1st 2 of the same length belonging to different chromosomes providing evidence to presence of duplicates maybe.

On a side note:

- the fact that the same protein is found in 4D of our species and 4D of *Aegilops tauschii* enforces an evolutionary interspecies link (also in our region which turned out to be on 4D, this is a bonus finding)

- It is also perceived in the other species, *Triticum durum*, that only has the A and B which is a sign of conservation of this protein among the genus of *Triticum*, implying its importance maybe in this organism.

All these blast results show that indeed this gene1 is highly likely to be associated with the *Anaphase-promoting complex subunit 11* protein, belonging to the 4D chromosome of our species, and there exist general agreement of the use of is full length (707aa) and is associated with a zinc-finger motif along with metal-binding activity (zinc most probably), with 100% match to the same protein

product from our own species. We shall see other results to see the validity of our assumptions

Augustus gene 2: (*protein length: 245aa*) *Against Triticum aestivum proteome*

Results firstly show 100% match of the first 245 residues of *Uncharacterized protein {ECO:0000313|EMBL:KAF7055961.1, ECO:0000313|EnsemblPlants:TraesCS4D02G339100.1}*, activity in DNA binding and transcription regulation, localized subcelluraly in the nucleus which might indicate its possibility to be a transcription factor. The aligned subject starts at 3rd residue, protein of length 247 indicating a possible mistake in the prediction of the gene length by AUGUSTUS.

The other 2 hits also have high % match (>95), same query length and position mapped but differ only in the start position of the subject protein and the chromosome location (do not map in 4D) suggesting possible duplicates.

Aegilops tauschii

Only one significant hit, same position as found previously 1-245, same exact annotation of the *Uncharacterized protein* with transcription involving function found on chromosome 4D of this species (showing evolutionary conservation).

Triticum durum

Same results almost, same positions are aligned (1-245), same functional annotation, compartmentalization (in nucleus) for 2 hits on respectively 5b and 4a chromosomes (near 4d position wise on whole genome). Also this uncharacterized protein provides evidence on the structural annotation of these 245 residues of our genomic region done by AUGUSTUS

Thus the 2nd prediction of AUGUSTUS is also highly likely to be validated, with provided proof on conservative 245 residues involved in dna binding activity

Fgenesh gene 1 & 2 Show absolutely no significant results in each of the species, one of them no hits at all, those that have have a higher proportion mismatches than matches, high e value and % id <40. Thus no validation of these gene structures predicted by Fgenesh

Fgenesh gene 3 (length: 621aa) This one provides interestingly similar results that also enforce our previously established hypothesis in the first Augustus gene:

Triticum aestivum

5 hits above 92%, all of them against the same protein (the *Anaphase-promoting complex subunit 11*)
Localized on chromosome 4D, matching the same length of our query (make sure) 1-621 in all 5 hits to different positions of the subject starting either at 67th or 76th residue with different alignment gaps and mismatch patterns

a more subtle hypothesis then having the same exact protein of different versions in the same place is having it align to the same protein entry, but we can not assume that especially that each of these 5 proteins has a different UniProt ID

Aegilops tauschii

These show similarity to AGUSTUS gene 1 results against this species' proteome. 4 hits of exactly 99.345% identity, the length is similar to previously reported (1-621 of query except the last one is 1-620) showing matches to respectively: *VWFA domain-containing protein* & *Anaphase-promoting complex subunit 11* (x2)
Also all metal bonding activity and present on 4D chromsome of this genome (matches with Augustus prediction)

Triticum durum

These results show top 3 >92% id with Anaphase-promoting complex subunit 11, also 1-621 against 87-707 of 2 subjects and 87-670 of the 3rd one. Thus promoting the same resulting conclusion

For this gene Fgenesh3, we would like to refer back to the analysis for Augustus gene 1 as something interesting is happening:

As previously stated from gene prediction results, these 2 predicted genes might be attempts to map the same gene, which is validated here through their mapping of Anaphase.. with high %id emphasising this segment's functionality

we can notice that August's prediction match the 1-707 of subject while Fgenesh start at either 67 or 76th residue of the subject, both matching the length of the predicted genes length (707 vs 621 residues), we can comment that Fgensh has a truncated prediction at the 5' UTR which is supposed to be part of the product. (We can say that the 621 aa residues have been well predicted in the coding region, there might be a truncated chunk at the beginning of the protein as predicted by Fgenesh as the matches are late to start on subject proteins and when put in comparison with the length and alignment of Augustus's)

Fgenesh gene 4 (length: 247aa)

Finally, we have here in *Triticum aestivum* proteome, 3 hits with 100% id, 247 residues of the query matching the same length of the subject, all of them are *Uncharacterized protein* but with same identifiers involved with ECO and such (also identifiers relatd to DNA-binding) with the same functional annotation, teh 100% match is with subject on 4D teh other 2 are respectively 4B and 5A which are closest to 4D in position, with the same exact alignment pattern, no mismatches, no gaps, and the same exact length of the protein.

2 further hits in *Triticum durum* and 1 in *Aegilops tauschii* show the same results.

Worth noting this matches with subject protein's length and further validates Augustus results, but even better with teh 2 first missing residues that we have mentioned before present here, thus highly suggesting the presence of a coding region here.

Transcriptome

still testing, might remove later

The *European Nucleotide Archive (ENA)* comprises a large collection of sequencing data from raw sequences to assembly to functionally annotated ones. While looking for transcriptome studies for *Triticum aestivum* we find several projects (Total= 22, in this table[7])

TSA stands for Transcriptome Shotgun Assembly

One of them is published by Xiao et al. (2013) in BMC Genomics [8]. They have performed short read RNA-seq using Illumina Hi-Seq tech, and deposited the project's raw reads on the SRA database, project `SRX212270`. We will use

[7]

Accession	Description
GAEF01000000	Triticum aestivum, TSA project GAEF01000000 data
GAJL01000000	Triticum aestivum, TSA project GAJL01000000 data
GBKH01000000	Triticum aestivum, TSA project GBKH01000000 data
GBKI01000000	Triticum aestivum, TSA project GBKI01000000 data
GBKJ01000000	Triticum aestivum, TSA project GBKJ01000000 data
GBKK01000000	Triticum aestivum, TSA project GBKK01000000 data
GBZP01000000	TSA: Triticum aestivum, transcriptome shotgun assembly.
GDTJ01000000	Triticum aestivum, TSA project GDTJ01000000 data
GEUX01000000	Triticum aestivum, TSA project GEUX01000000 data
GEWU01000000	Triticum aestivum, TSA project GEWU01000000 data
GFFI01000000	TSA: Triticum aestivum, transcriptome shotgun assembly.
GIJS01000000	Triticum aestivum, TSA project GIJS01000000 data
GILY01000000	Triticum aestivum, TSA project GILY01000000 data
GIXT01000000	TSA: Triticum aestivum cultivar TcLr19 isolate leaf, transcriptome shotgun assembly.
GJAR01000000	TSA: Triticum aestivum cultivar Avocet R, transcriptome shotgun assembly.
GJUY01000000	TSA: Triticum aestivum, transcriptome shotgun assembly.
HAAB01000000	Triticum aestivum, TSA project HAAB01000000 data
HCEC01000000	TSA: Triticum aestivum
HCED01000000	TSA: Triticum aestivum
IAAK01000000	TSA: Triticum aestivum, transcriptome shotgun assembly.
IAAL01000000	TSA: Triticum aestivum, transcriptome shotgun assembly.
IAAM01000000	TSA: Triticum aestivum, transcriptome shotgun assembly.

[8]The Galaxy server used for some calculations is partly funded by the German Federal Ministry of Education and Research BMBF grant 031 A538A de.NBI-RBC and the Ministry of Science, Research and the Arts Baden-Württemberg (MWK) within the framework of LIBIS/de.NBI Freiburg.

this as trial to explore how we can validate using Whole Transcriptomes before optimizing our choice.

Trial 1: blasting against transcriptome

As a first attempt, due to the high memory requirement (*e.g.,* one of them is 15GB of reads), we have tried performing BLAST on ncbi's server against this whole transcriptome in [9], with default parameters (can perform it here by just adding the region8 fasta file). The default search gave no significant results, we will try to relax the paramters (BLOSUM45 and lowering penalties, accepting lower thresholds...)

Trial 2: downloading the WTS data

We will try downloading the reads of [10] to see how to manipulate such a large file. Since it surpasses the threshold to download a file from SRA webserver (which is 5GB), we will download it using `sra-toolkit`.
While running out of time and memory, we will try doing that using Galaxy[11][12].

Trial 3: Analysis

Working on galaxy, first retrieve the SRA accession number from the project, tools > Get data > EBI SRA, copy the accession number and get the fastq in galaxy. After loading them (paired end so 2 fastq) > fastq groomer, to make sure the fastq format fits Galaxy's requirement and make it run. Meanwhile > FastQC to make sure the quality of the transcriptome is good or whether it's better to take another set of reads.

We will try now mapping: using Tophat2, we will map the reads to the reference genome of *Triticum aestivum* (available on ENSEMBL) to see how many reads are mapped and how many are not. We have taken the reference genome using

Trial 4: visualization

trying to perform RNA-seq aln and viz using IGB

[9]Xiao, J., Jin, X., Jia, X., Wang, H., Cao, A., Zhao, W., ... & Wang, X. (2013). Transcriptome-based discovery of pathways and genes related to resistance against Fusarium head blight in wheat landrace Wangshuibai. BMC genomics, 14, 1-19.

[10]Xiao, J., Jin, X., Jia, X., Wang, H., Cao, A., Zhao, W., ... & Wang, X. (2013). Transcriptome-based discovery of pathways and genes related to resistance against Fusarium head blight in wheat landrace Wangshuibai. BMC genomics, 14, 1-19.

[11]The Galaxy platform for accessible, reproducible, and collaborative data analyses: 2024 update Nucleic Acids Research, gkae410 doi:10.1093/nar/gkae410

[12]The Galaxy server used for some calculations is partly funded by the German Federal Ministry of Education and Research BMBF grant 031 A538A de.NBI-RBC and the Ministry of Science, Research and the Arts Baden-Württemberg (MWK) within the framework of LIBIS/de.NBI Freiburg.

cDNA

cDNA (complementary DNA) is a single-stranded DNA synthesized from a messenger RNA (mRNA) template in a reaction catalyzed by the enzyme reverse transcriptase. It is thus synthesized from the mRNA template, it can be used to study the gene expression in a cell, as it is a copy of the mRNA, and can be used to study the gene expression in a cell. It's a representation of a gene's transcript. On Ensembl Plants, we can find the cDNA of *Triticum aestivum here on this ftp site (click link).* There is one fasta file containing all of the genome's cDNA sequences, with a particular header format. To make the process more easily computable, we wrote a bash script to filter the cDNA sequences of the chromosome 4D, and save them in a separate file.

also downloaded pep, CDS, ncRNA and annotations (gff)

Transposable Elements (TEs)

Final annotation

Gene 1 of Augustus (predicted protein) which has a length of 707aa has shown to perfectly align with a subject of the protein **Anaphase-promoting complex subunit 11** which also has the same length, so this is our first final annotated gene, with positions as reported by AUGUSTUS:

Feature	Start	End
gene	6226	10861
transcript	6226	10861
exon	6226	6762
start_codon	6532	6534
initial	6532	6762
terminal	8714	10606
intron	6763	8713
CDS	6532	6762
CDS	8714	10606
exon	8714	10861
stop_codon	10604	10606
tts	10861	10861

What's Anaphase-promoting complex subunit 11?

keywords: *Metal-binding, Zinc, Zing-finger, Anaphase-promoting complex subunit 11, RING*
This is an unreviewed protein annotation (TrEMBL) with score 1/5, no structure has been experimentally determined which weaken its annotation status.

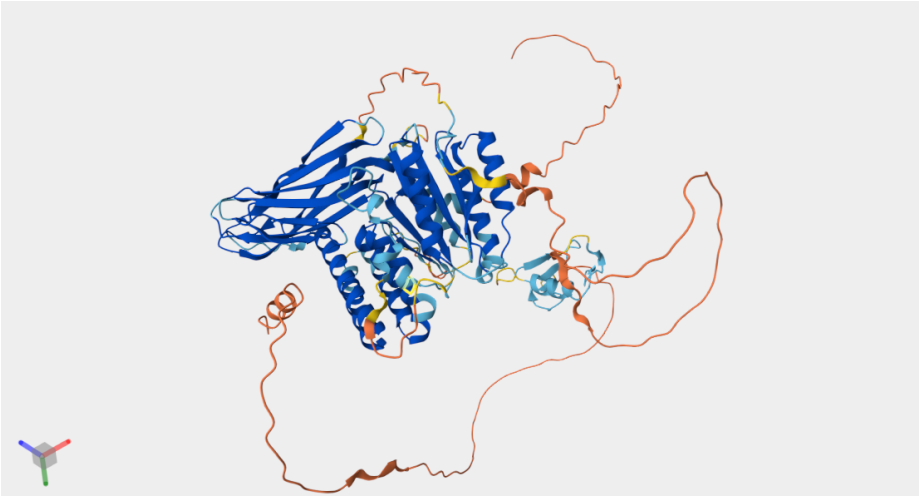


Figure 9: Anaphase-promoting complex subunit 11 predicted structure from AlphaFold

Supplementary

- Whole Genome (all 7n chr) of *triticum aestivum* on ENSEMBL : https://ftp.ensemblgenomes.ebi.ac.uk/pub/plants/release-60/gff3/triticum_aestivum/
- ENSEMBL in general : https://plants.ensembl.org/Triticum_aestivum/Info/Index
- ENA: https://www.ebi.ac.uk/ena/browser/view/Taxon:4565
- SRA: Sequence Read Archive, repository for seq data
- RNAseq reads fetch and viz: youtube video
- RefSeq: reference sequence v2.1 here, link to acces the dataset is *here*
- downloading a proteome of a species from uniprot, EMBL-EBI training course
- Chromosome 4D annotations in GFF *ftp link*