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Understanding transcription factors and how they affect metabolic processes in cucumber sex determination

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Abstract: Plant reproduction is a very important process on Earth from the perspective of biodiversity, biomass gain and crop productivity. It is therefore important to understand sex determination process and many researchers are investigating the molecular basis of this phenomenon. However, information on the influence of transcription factors (TFs) on this process is limited, although cucumber is a model plant in this regard. In the present study, based on RNA-seq data for differential gene expression (DEG) analyses, we aimed to investigate the regulatory TFs that may influence the metabolomic processes in the shoot apex containing the forming flower buds. Therefore, a robust TF database was established for the B10v3 cucumber genome. Sex-specific interactome network maps were generated, indicating the regulatory TFs by their effects on DEGs and further on processes leading to the formation of different sex flowers. The network analysis identified major families of regulatory TFs. The most abundant families were: MYB, AP2/ERF, NAC and bZIP, and those with the greatest impact on developmental processes were identified, namely the AP/ERF family, followed by DOF, MYB, MADS and others. Thus, the central nodes and key regulators in the networks were identified with respect to male, female and hermaphrodite. Here, we proposed the first model of the regulatory network of TFs that influences the metabolism of sex development in cucumber. These findings may help to understand the molecular genetics and functional mechanisms underlying sex determination processes.

Keywords: transcription factors, interactome network, sex development, sex determination, Cucumber (*Cucumis sativus*); metabolomic processes

1. Introduction

Cucumber (*Cucumis sativus*) is a globally significant vegetable crop and is also recognized as a model organism for exploring the intricacies of plant sex determination, encompassing male, female, and hermaphrodite forms. Although the process of sex determination in cucumbers is currently the focus of numerous scientific investigations, the underlying mechanisms of sex determination in this species remain incompletely understood.

The knowledge about the transcription factors that influence the metabolic processes involved in sex determination is also very limited. It will be very interesting to see which transcription factors and which metabolic processes are involved in processes of sex determination in cucumber. With the increasing amount of omics data such as sequenced genomes and transcriptomes, there is a strong basis and the opportunity to construct interactome networks that indicate the influence of regulatory TFs on selected processes.

Among the most well-known and described reference genomes of *Cucumis sativus* are: 9930 - Chinese line [1] Gy14 North American line [2] and B10v3 European line [3] Recently, genomes were compared using structural data presented in databases as well as previously reported experimental data. It was shown that the B10v3 genome is the longest due to 342.5 Mbz assembled [4]. Among others, in order to have the most complete picture of the relevant elements, in this study we focused

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on this version of the genome for further analyses. Understanding how genomes are organized is the basis for insight into the functioning of organisms. Knowledge of regulatory mechanisms and their links to metabolic processes is an important part of the interactions that control gene action.

Transcription factors are proteins that control the activity of gene regulatory networks and cell type specification. They represent a class of essential regulatory proteins that are critical for controlling gene expression and modulating various physiological processes in plants, including: development, hormone signaling, and stress responses. TFs play an important role in the regulation of complex metabolic pathways in response to environmental and physiological signals. They are key regulators of plant primary and secondary metabolism, that produce a large number of specialized metabolites with a wide range of functions and applications [5,6]. However, the inferred function of TF can be influenced by the genomic context in which it occurs. Each family of TFs possesses a specific DNA binding domain that recognizes a unique DNA sequence. In addition, knowledge of the genome can be useful for identification of novel TFs [7]. TFs bind to sequences of DNA, usually to motifs in the promoters of their target genes. Together with other proteins, such as transcriptional regulators (TRs) they regulate gene transcription [8]. The regulatory mechanism underlying gene expression mediated by TFs relies upon the fundamental process of binding to *cis*-regulatory elements located within gene promoters [9] influencing the expression of nearby genes. This regulatory mechanism plays an important role in orchestrating gene expression in plants [5].

In recent years, the identification and characterization of TFs have been made possible by the development of numerous databases, including iTAK [6] and PlantRegMap [10] which encompass 197 and 165 plant organisms, respectively. These databases contain detailed descriptions of broadly classified TF families for each species, thereby facilitating their efficient exploration. Furthermore, the iTAK database offers supplementary information on TRs that function by interacting with the basal transcriptional apparatus, which includes TFs [6]. This additional information is particularly valuable in expanding our understanding of the intricate mechanisms underlying gene expression regulation in plants. By leveraging the comprehensive information provided by these reference databases, researchers can efficiently search for TFs within the results of a transcriptome study experiment and examine their interactions with other components in the system being investigated. This provides a valuable framework for investigating the complex regulatory networks underlying various biological processes in plants among which there is sex determination in cucumber.

In the present work, the objectives of the analyses were: (1) to identify and localize of TFs in the B10v3 cucumber genome according to known TFs in the databases, (2) to update functional characteristics of differentially expressed genes (DEGs) pointed in RNA-seq analyses regarded to sex determination, (3) to search for regulatory TFs that may influence genes correlated with sex expression (4) to establish an interactome map of regulatory TFs and their target genes that have been identified as relevant in sex determination analyses (5) to examine which metabolic processes are associated with regulatory TFs that form a sex-specific interactome and have the impact on the DEGs.

In this paper, we present the world's first-ever interactome map of transcription factors influencing metabolic processes linked to sex determination in cucumber, providing their functional characterization. We proposed multi-omics to integrate data and gain a complex view on the interplay between cell signaling and gene regulation in regard to specific sex in plants.

2. Materials and methods

The work presented in the publication consisted of two steps carried out: the search for TFs in the B10v3 cucumber genome [3] and the functional analysis of RNA-seq data from an experiment comparing leaves and shoot apex expression between cucumber lines differing in sex [11]. A schematic representation of the analytical methodology employed for each of these discrete steps is provided in Figure 1.

Figure 1. Diagram showing the analysis steps performed.

2.*1 Finding the transcription factors in the B10v3 genome.*

To identify TFs in the B10v3 genome, data from two databases were used: PlantRegMap and iTAK. The PlantRegMap database contains information on plant TFs and also provides available software to detect TFs in the genome. The iTAK database contains information on TFs, TRs and protein kinases (PKs), as well as software for their detection in a given datasets. To detect the TFs in the B10v3 genome dataset, the longest amino acid sequences per gene were used as an input for TF identification. In order to work with the PlantRegMap database it was necessary to have gene identifiers that were consistent with the identifiers contained in the database. This required the translation of the protein identifiers from the B10v3 genome to those in the database which are the Gy14v1 cucumber genome identifiers. For this purpose, the "ID mapping" tool that is available on the database server of PlantRegMap was used.

2.2 Tanscription factors among the results of RNA-seq experiments

TFs were searched among DEGs (genes with statistically significant differential expression from RNA-seq experiment) from shoot apex between cucumber lines differing in sex [11]. We analyzed DEGs in the leaves vs shoot apex (LvS) and in the shoot apex based on the following three comparisons of flower sex types: female vs male (FvM), female vs hermaphrodite (FvH), and male vs hermaphrodite (MvH). In the previous experiments, the sequencing reads were mapped to one of the first versions of reference cucumber genome. Therefore, it was necessary to update information and connect them using BLAST algorithm with the gene identifiers of the newest version of the genome - B10v3 [3]. Using the results of the RNA-seq experiment together with the information TFs within the B10v3 genome (from the previous step 2.1), DEGs were assigned to the TFs, TRs or PKs family, according to PlantRegMap and iTAK database.

2.3 Ontology analysis among differentially expressed genes

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The ontology of the DEGs was examined using the GO Term Enrichment tool from the PlantRegMap database to gain further insight into the metabolic processes which could differ cucumber lines with the varying sex. This tool helps to identify significantly overrepresented GO terms or the parents of these terms in the selected gene set. This analysis was based on the DEGs identified in the LvS and FvM, FvH, MvH comparisons [12].

2.4 KEGG pathways enrichment analysis in DEGs

DEGs were subjected to KEGG pathway analysis to identify enriched KEGG terms. Gene identifiers from the *Cucumis sativus* 9930 genome were required to use the KEGG database. To obtain these identifiers, the BLASTP program was used to search for the peptides coded by the DEGs among the protein database for 9930 genome datasets. The ShinyGO server [12] was used to perform the KEGG pathways enrichment step. An annotation for the B10v3 genome has been added for each of the genes which are part of the detected enriched KEGG terms.

2.5 Regulatory Transcription Factors enrichment in PlantRegMap for DEGs

The 'TF enrichment' tool in the PlantRegMap database was used to determine the enrichment of regulatory TFs which interact with the DEGs. Retrieved regulatory TFs were identified from literature and ChIP-seq data and also inferred by combining TF binding motifs and regulatory element data, according to PlantRegMap database. Both regulatory TFs and targeted DEGs were used to build the interactome networks. They were prepared using the networkD3 library in the R programming language [13]. The study determined the effect of the interaction relevance of the TF families on the DEGs based on the constructed networks.

3. Results and discussions

3.1 Transcription factor search results in the B10v3 genome.

In order to get a complete view of the TFs across the genome, we carried out a whole genome analysis using B10v3 as a reference. Using the PlantRegMap and iTAK databases, it was possible to find TFs in the B10v3 cucumber reference genome. The results of the matching between the databases differed slightly, but for the most part remained consistent with each other. Based on the consensus rules for TF prediction and classification with the use of data from the PlantTFDB database, iTAK database was created [6]. Annotations from the iTAK database were therefore used in the analyses. Using the "TF prediction" tool available in the PlantRegMap database, 1 045 TFs were assigned to the searched amino acid sequences. The same input file was used to search for TFs in the iTAK database, allowing for annotation of 1 082 TF families, 1 355 TRs and 656 PKs in the B10v3 genome. Of the 16 104 sequences of the B10v3 genome, 14 269 sequence identifiers were assigned from the PlantRegMap database (Supplement S1). TF search results from the PlantRegMap and iTAK databases were added to the B10v3 genome annotation and supplemented with TR and PKs search results. The resulting B10v3 genome annotation is provided in Supplement 2. For the results obtained using the PlantRegMap and iTAK databases, the percentage of detected TFs per family is shown in Figure 2. The prepared annotation of the B10v3 genome, supplemented with information on genes encoding TFs, RFs and PKs, enriches the knowledge of the genome of B10v3 cucumber line. The number of TF families detected in the cucumber genome corresponds to TFs detected in other plants [14]. The distribution of the identified genes itself is consistent with the factors detected in other plants, where the main TF families are: MYB, bHLH, NAC, WRKY [15].

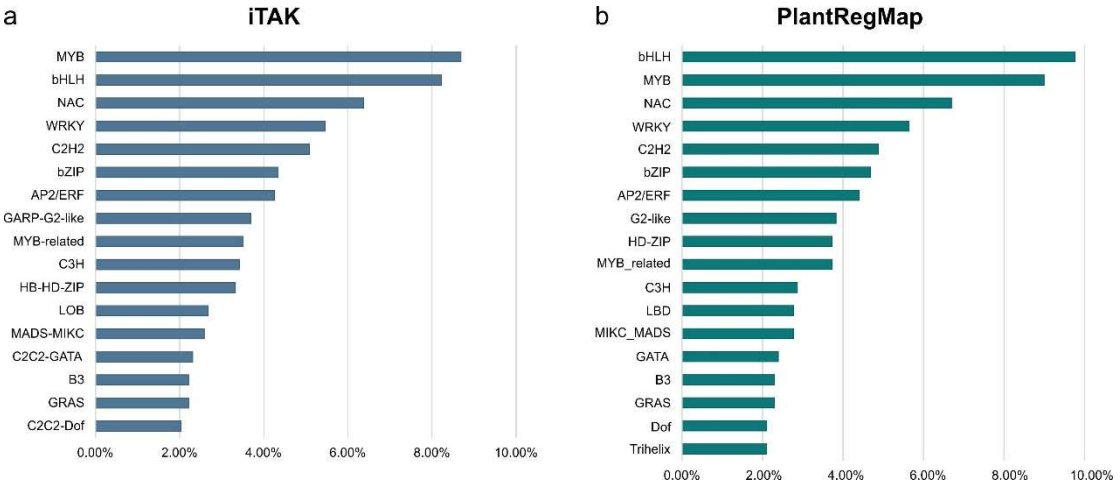

Figure 2. Transcription factor families detected in the B10v3 genome by the iTAK (a) and PlantRegMap (b) databases, which account for more than 2% of all identified TFs.

3.2 Transcription factor among DEGs

TFs have been identified among sex specific DEGs sets from RNA-seq analyses [11]. The results of the RNA-seq experiment supplemented with annotation of TFs, TRs, PKs can be found in Supplement S3. Among DEGs from the tested comparisons: LvS, FvM, FvH, MvH, the 8,7%, 10,77%, 5,88% and 10,91% of TFs were detected, respectively (Table 1).

Table 1. Number and percentage of transcription factors found in the list of genes with significantly differential expression for LvS, FvM, FvH, MvH comparisons.

Comparison	Number of DEGs	Number of TFs found for the comparison:	% of all DEGs
LvS	2852	248	8,70%
FvM	260	28	10,77%
FvH	36	2	5,88%
MvH	55	6	10,91%

The largest number of TFs was detected for the LvS comparison (8,70%). However, this number is due to the largest number of DEGs detected between the leaf and shoot apex and contains genes responsible for the transition from the vegetative to flowering phase. Number of DEGs in the shoot apex among male, female, and hermaphrodite lines was significantly lower, while the percentage of TFs detected for these comparisons remained similar. In comparison MvH, where six TFs were detected, half of them were the MADS-MIKC family. The other assigned families were HB-WOX, NAC, C2C2-YABBY. For comparison FvH, in which only two TFs were detected, the bHLH and C3H families were defined. The following Figure 3 and Figure 4 show graphs with the highest number of the TFs detected in the FvM and LvS comparisons, respectively. For the FvM and MvH comparisons the largest number of differentially expressed factors belonged to the MADS-MIKC family. This represents a significant increase in the proportion of these TFs relative to their contribution to the whole genome. When comparing LvS, the TFs that were found in the highest abundance, i.e., bHLH, MYB, NAC, and C2H2, correspond in abundance to the distribution of TFs across the genome. TFs of the MADS-MIKC family in this comparison also represent an increased proportion in the number of 16 differentially expressed TFs relative to their presence in the reference genome. MADS TFs are a family of DNA-binding proteins that play an essential role in various plant developmental processes, especially floral organ identity and differentiation [16,17] additionally controlling the expression of genes that determine the identity and morphology of sepals, petals, stamens, and carpels. The MADS TFs in cucumber are similar to those in other plants, as they are also involved in the flowering time regulation and the floral organs developement [18]. The detection of the MIKC-MADS family as the

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most abundant among the TFs detected directing us towards linking MADS to the ABC model of flower development [19].

In addition, the presence of eight differentially expressed TFs of the AP2 family is important notification, due to the link between sex determination processes and ethylene metabolism [20].

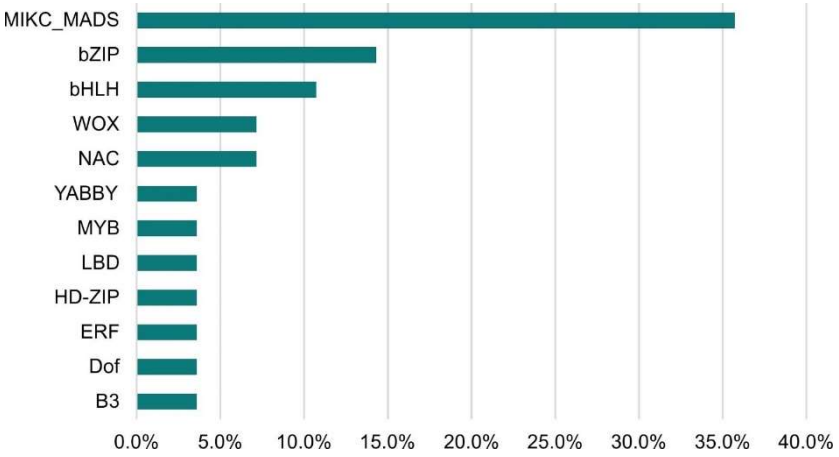

Figure 3. Percentage of transcription factors families among differentially expressed genes in FvM comparison.

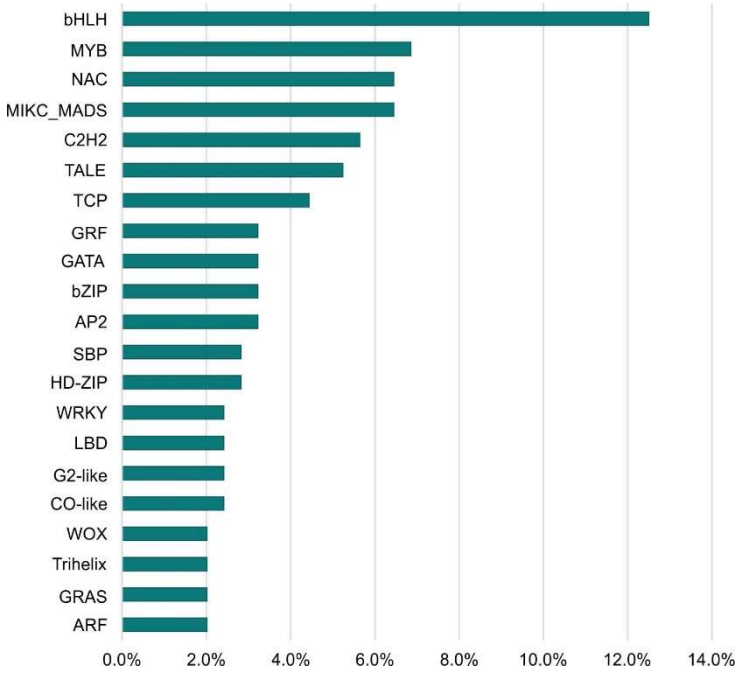

Figure 4. Percentage of transcription factors among differentially expressed genes in LvS comparison.

3.3. Ontology analysis among differentially expressed genes

For DEGs, an overrepresentation of GO terms was found in all comparisons: LvS, FvH, FvM and MvH, what is presented in Fig. 5. For FvH, most of the GO terms were related to processes like pollen sperm differentiation, male gametogenesis, or microgametogensis differentiation, indicating significant relationships to processes involved in sex determination and male organ formation. In this comparison, the female organs are formed in the female as well as in hermaphrodites, while the male organs are present only in hermaphrodite flower.

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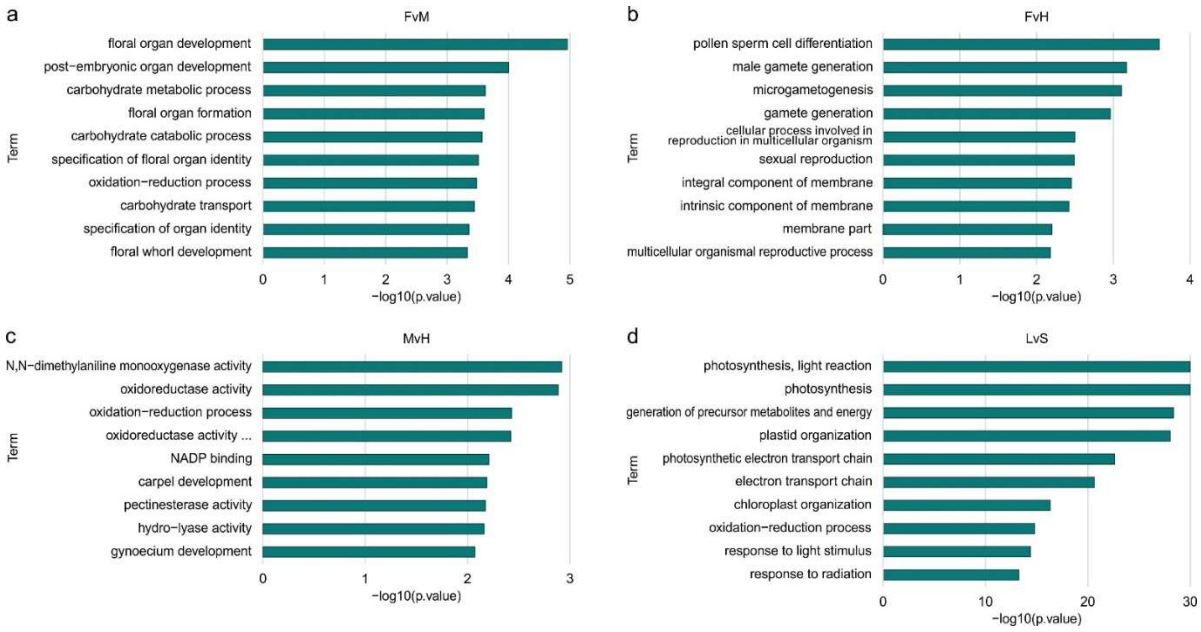

Figure 5. Box plots of significantly enriched GO terms for genes with significantly differential expressions for LvS, FvM, FvH, MvH comparisons.

In the FvM comparison, the most significant enrichment concerned genes that are involved in the flower formation developmental processes, overall organ development and carbohydrate metabolism processes.

Processes related to enzyme activity: monooxygenase, oxidoreductase, hydrolase or pectinesterase were the most enriched in MvH comparison. Genes involved in the development of the carpels and gynoecium were also enriched. In this comparison, the female organs are formed in the hermaphrodite flowers but in male flowers this organ is inhibited in the growth, thus the difference connected to these group is expected. When comparing LvS, the enriched processes differed significantly from the other comparisons. The highest enrichment in this case was for processes related to photosynthesis, light response, plastid or chloroplast organization. Comparing a vegetative organ such as the leaf with a generative organ such as the whole structure of shoot apex with small floral buds, indicates which genes and processes differentiate these two organs mostly. The number of DEGs is the highest in the LvS comparison and significantly exceeds the number of DEGs in the other comparisons. The analysis of the ontology network (Supplements S4-S7) shows that those processes are significantly enriched and are marked in red and yellow. For the FvH and MvH comparisons, the created ontology networks are significantly simpler than the other: FvM and LvS comparisons, which is due to the smaller number of significantly DEGs identified. FvH comparison represents the most enriched final processes, converging to a single final process of pollen germ cell differentiation. Similarly, for the MvH comparison, the structure of the ontology converges on final terms describing gynoecium development and carpel development, although a separate branch indicating metabolic processes and a final term describing oxidation and reduction processes are additionally described. In comparison the FvH ontology network is much more extensive, where three main branches can be observed. The first corresponds to floral organ formation processes, the second indicates metabolic processes taking place while the third describes enriched processes related to transport. In the case of ontological terms describing organ development, processes such as floral organ development and floral organ formation have the greatest enrichment. In addition, we can distinguish terms describing gynoecium development and carpel development. The processes responsible for oxidation-reduction and carbohydrate metabolism have the greatest enrichment within metabolic processes. The different branches of ontology converge to final terms describing processes related to metabolism of pectin, salicylic acid, inositol and fatty acids. A separate branch of the network describes processes related to transport of carbohydrates, saccharides and sucrose.

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The next step of the analysis was to check whether there were DEGs in the enriched GO term that were TFs. For this purpose, we checked all differentially expressed TFs in the sex specific comparisons. In Figure 6, the frequency of TFs among DEGs for each ontology term is shown for FvM comparison. It can be seen that TFs are responsible for floral developmental processes, carpel development, and organ formation. This indicates the actual involvement of TFs in processes linked to the plant's sex development. No TFs were detected for the enriched ontology terms in the FvH comparison. For the MvH comparison, two TFs involved in gynoecium and carpel development processes were detected among the enriched ontology terms. They are also directly related to issues of plant sex development which, as can be seen, represents the relevance of TFs in this process. The LvS comparison is the most abundant. Therefore, it consists of the largest number of TFs. The ontological terms to which the TFs were assigned were in relation to metabolic processes and biosynthesis.

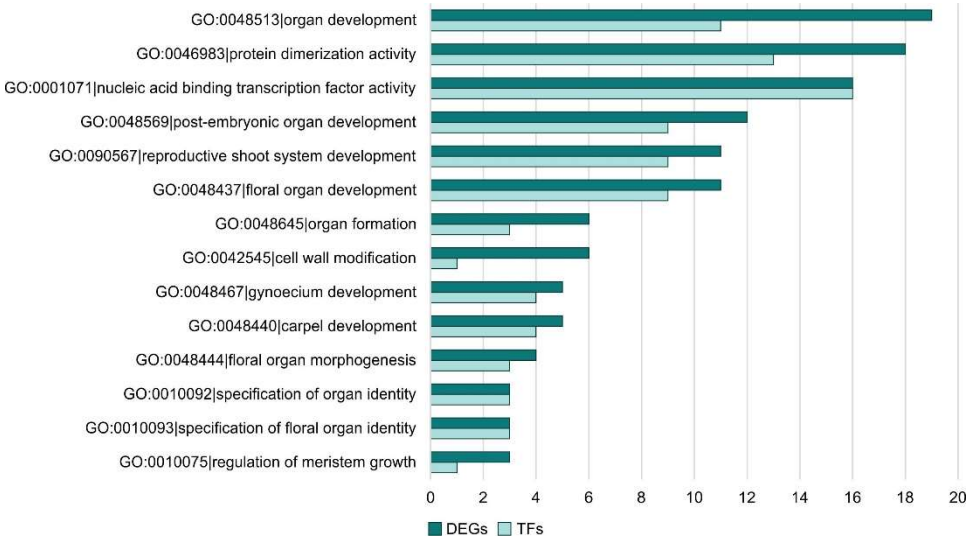

Figure 6. The number of TFs among DEGs for FvM comparison regard to ontology terms.

3.4 *KEGG enrichment results in DEGs*

The enrichment analysis of the KEGG pathways, performed for DEGs (LvS, FvM, FvH, and MvH comparisons) allowed for a better understanding of the related functions and networks involved in related to sex determination processes. The KEGG enrichment results are shown in Figure 7. No statistically significant results were obtained for the FvH comparison. For the FvM comparison, the highest enrichment is related to the ABC transporters pathway, which refers to the ATP-binding cassette (ABC) transporters. For both the FvM and MvH comparisons, statistically significant enrichments were found for the pentose and glucuronate interconversion pathways. When comparing LvS, the most significant were pathways included photosynthesis and metabolic processes. The annotated results of the KEGG analysis are presented in Supplement S8. A search for transcription factors among the genes in the KEGG enrichment pathway did not identify any TFs. Only protein-coding genes were present in the KEGG pathways found in the analysis. However, similar to GO analysis, significant differences in overrepresented pathways could be observed between the generative (FvM, MvH comparisons) and vegetative (LvS comparison) organs.

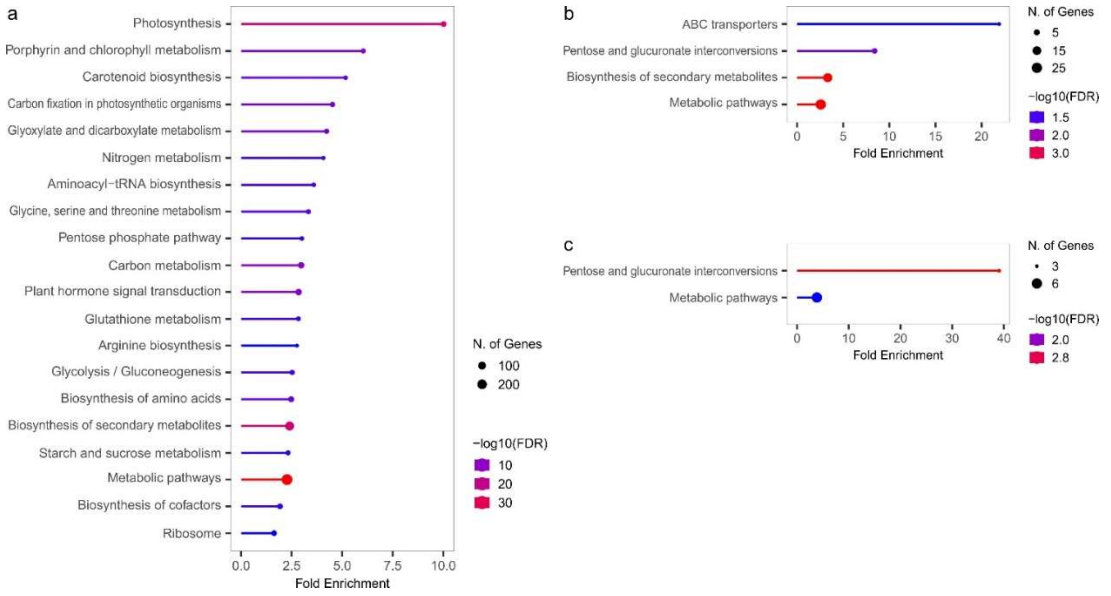

Figure 7. Graphs showing enriched KEGG pathways for LvS (a), FvM (b) and FvH (c) comparisons.

3.6. Regulatory Transcription Factors influencing DEGs.

The study of flowering in cucumber is crucial due to its significant economic importance and vulnerability to both: endogenous and exogenous factors. The impact of these factors collectively determines the expression of genes, which in turn is influenced by the activity of various TFs. The regulatory TFs were assigned to families and functionally curated.

Our study shows the interaction of regulatory TFs and their influence on DEGs thus, taking together, we can answer the question: what TFs influence flower morphogenesis at early stages of growth.

As a result of the TF enrichment program in the PlantRegMap database, a list of enriched regulatory TFs was obtained for each of the FvM, FvH, MvH and LvS comparisons. The retrieved regulatory TFs were annotated according to B10v3 information data sets. (Supplement S9). Analysis of the detected TF families revealed that the NAC family was the most abundant family detected for the FvH and MvH enriched comparisons. Furthermore, genes encoding TF families such as bHLH and MYB showed a higher frequency of detection. Notably, a greater number of enriched TFs were identified in the FvH and MvH comparisons compared to the FvM comparison. For each of the comparisons considered, enriched TFs were detected for a significant number of TF families. The LvS comparison showed the highest number of enriched TFs, with MYB, bZIP, bHLH and DOF being the most frequently detected families.

Created interaction maps between regulatory TFs and their targeted DEGs are highly complex and have therefore been presented in the form of interactive networks available in HTML files (Supplements S10-S13). The advantage of such a network presentation is that it is possible to view the gene of interest together with the genes with which it interacts (DEGs or regulatory TFs), and to read their annotation. A static image of the networks for FvM, FvH and MvH comparisons is presented in Figure 8.

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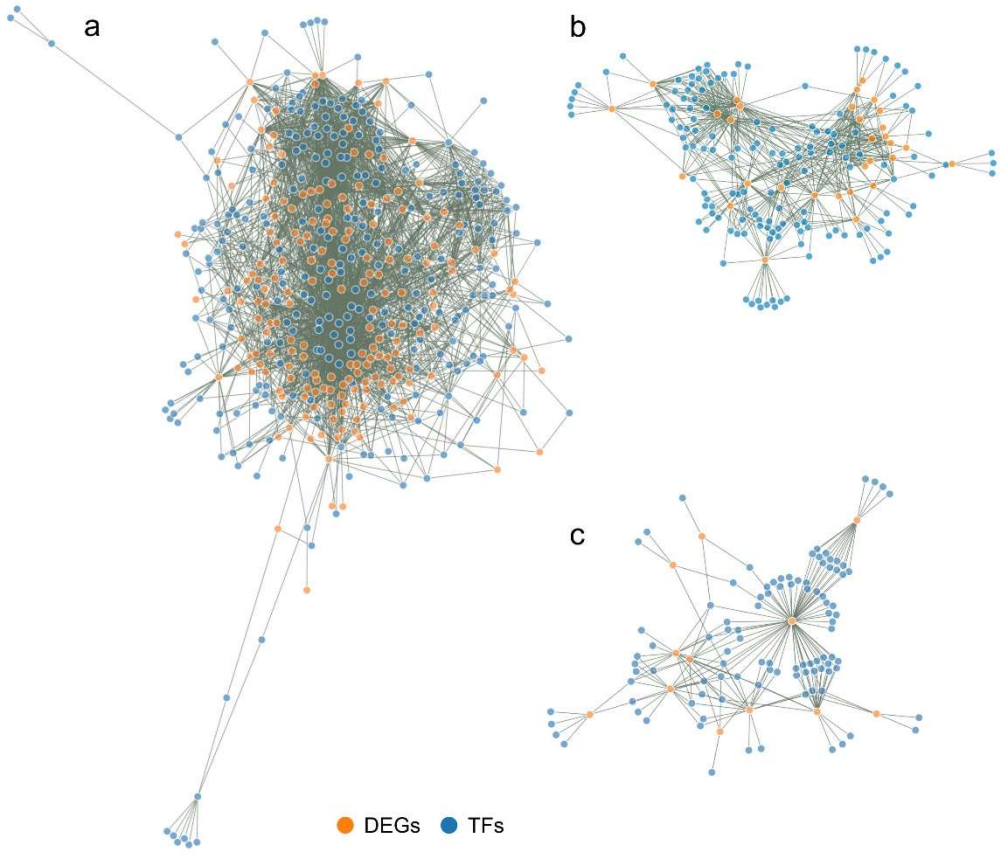

Figure 8. Static image of interactions networks between transcription factors and differentially expressed gene targets in FvM comparison (a), FvH comparison (b) and MvH comparison (c). Orange color indicates DEGs, while blue color indicates regulatory TFs.

Between FvM, the number of DEGs is significantly higher than that for FvH and MvH comparisons. The FvM interaction network thus contains many more connections, showing that the male and female lines have significant differences in expressed genes. The FvH and MvH lines are thus a simplified model as the number of differentially expressed genes is much smaller but refers to processes associated with sex variation.

The resulting interaction networks between regulatory TFs and DEGs varied in complexity, what was based on the number of DEGs used to construct the network. The largest network was created for the LvS comparison consisted of 3029 nodes. The network for FvM comparison consisted 468 nodes, for FvH comparison consisted of 177 nodes, while for MvH comparison consisted of 191 nodes. The number of individual regulatory TFs and DEGs used to construct the networks is shown in Table 2. The smallest networks were created for the FvH and MvH comparisons, due to the flowers possessing a common element in the flower architecture. The FvM network is more developed due to flower architecture concerning distinction of the generative organ.

In the next step we grouped up regulatory TFs that influence DEGs into 34 families and performed functional characterization (Supplements S14). This allowed as to elucidate which and how the regulatory TFs influence DEGs and thus proteins involved in metabolic processes in lines varying in sex in cucumber. In order to check the force of influence of regulatory TFs families, the links between TFs and their targets were counted (Fig. 9).

Table 2. The number of DEGs and regulatory TFs that were used to create the interactome network for FvM, FvH, MvH, LvS comparisons.

	FvM	FvH	MvH	LvS
DEG	**186**	**31**	**35**	**244**
TF	**282**	**147**	**156**	**2785**

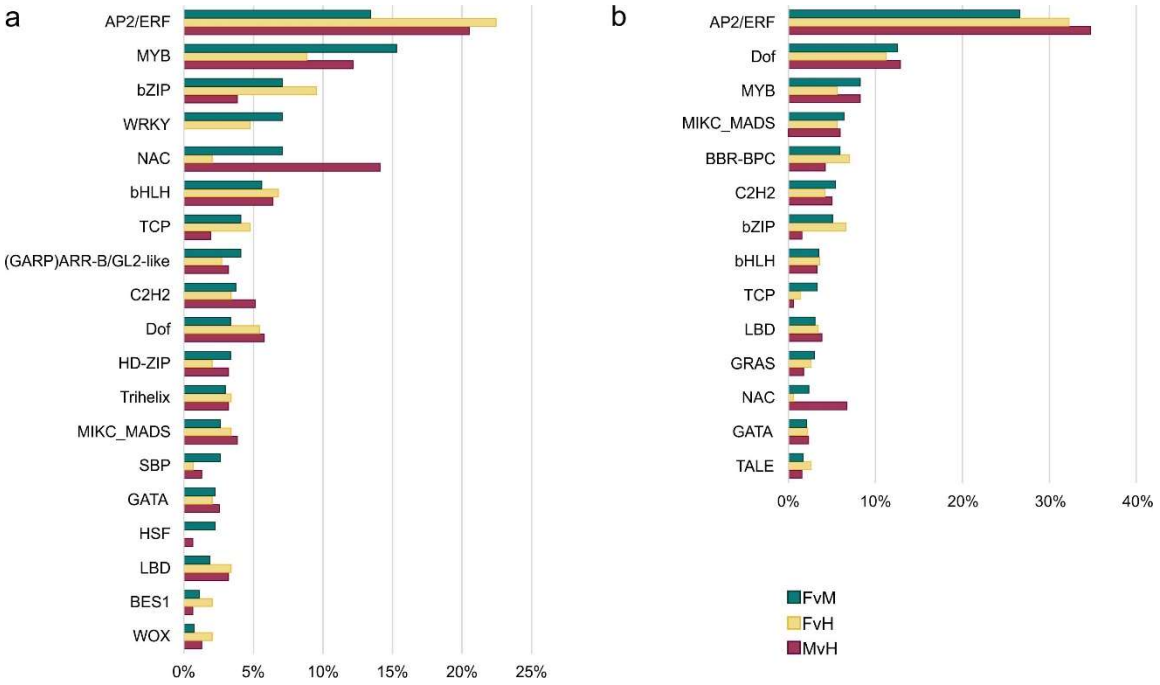

Figure 9. The number of nodes represents TFs from different family (a) and the number of edges to TFs family (b) among the different comparisons.

Table 3. Top 10 Number of TFs from established interaction networks that have the most connections to DEGs.

Comparison	Transcription factor	No. of edges
FvM	Cucsa.362960|MIKC_MADS|MADS box transcription factor	116
	Cucsa.277740|AP2|AP2-like ethylene-responsive transcription factor	109
	Cucsa.026600|BBR-BPC|GAGA-binding transcriptional activator	93
	Cucsa.307870|C2H2|Transcription factor IIIA	82
	Cucsa.102120|Dof|Dof zinc finger protein	81
	Cucsa.280310|GRAS|DELLA protein GAI	81
	Cucsa.213830|Dof|Dof zinc finger protein	76
	Cucsa.159750|BBR-BPC|GAGA-binding transcriptional activator	69
	Cucsa.341290|Dof|Dof zinc finger protein	60
	Cucsa.098430|Dof|Dof zinc finger protein	48
FvH	Cucsa.362960|MIKC_MADS|MADS box transcription factor	23
	Cucsa.026600|BBR-BPC|GAGA-binding transcriptional activator	21
	Cucsa.277740|AP2|AP2-like ethylene-responsive transcription factor	19
	Cucsa.307870|C2H2|Transcription factor IIIA	17
	Cucsa.159750|BBR-BPC|GAGA-binding transcriptional activator	14
	Cucsa.280310|GRAS|DELLA protein GAI	13
	Cucsa.353140|TALE|Homeobox protein knotted-1-like 1	13
	Cucsa.102120|Dof|Dof zinc finger protein	12
	Cucsa.213830|Dof|Dof zinc finger protein	12
	Cucsa.098430|Dof|Dof zinc finger protein	9

MvH	Cucsa.362960|MIKC_MADS|MADS box transcription factor	21
	Cucsa.102120|Dof|Dof zinc finger protein	17
	Cucsa.277740|AP2|AP2-like ethylene-responsive transcription factor	16
	Cucsa.213830|Dof|Dof zinc finger protein	12
	Cucsa.307870|C2H2|Transcription factor IIIA	12
	Cucsa.341290|Dof|Dof zinc finger protein	12
	Cucsa.026600|BBR-BPC|GAGA-binding transcriptional activator	11
	Cucsa.159750|BBR-BPC|GAGA-binding transcriptional activator	11
	Cucsa.136780|ERF|Dehydration responsive element binding transcription factor	9
	Cucsa.237150|ERF|Ethylene-responsive transcription factor ERF021	9
LvS	Cucsa.362960|MIKC_MADS|MADS box transcription factor	1579
	Cucsa.277740|AP2|AP2-like ethylene-responsive transcription factor	1485
	Cucsa.026600|BBR-BPC|GAGA-binding transcriptional activator	1324
	Cucsa.307870|C2H2|Transcription factor IIIA	1205
	Cucsa.280310|GRAS|DELLA protein GAI	1090
	Cucsa.102120|Dof|Dof zinc finger protein	1076
	Cucsa.213830|Dof|Dof zinc finger protein	1031
	Cucsa.159750|BBR-BPC|GAGA-binding transcriptional activator	1000
	Cucsa.353140|TALE|Homeobox protein knotted-1-like 1	920
	Cucsa.341290|Dof|Dof zinc finger protein	823

TFs are proteins that help to 'turn on' or 'turn off' certain genes by binding to the promoter, thereby regulating the functioning of the organism. The present study identified several TF families that majorly influence the expression of DEGs in male, female and hermaphrodite flowers. The most numerous family of regulatory TFs in all three networks were: AP2/ERF (total 101), MYB (73), NAC (44), bZIP (39) and bHLH (35) family. Other families were less numerous in the sum of the three comparisons (Fig 9). However, some TFs are specified only for one comparison, namely FvM: YABBY, LFY, SRS, EIL or only for to comparison of FvH and FvM, such as: WRKY, CPP, FAR1 and for other set, FvM and MvH - HSF family. In terms of edge numbers, that is, family interactivity, which can be translated into power to influence DEGs, the most numerous families totally in three networks were also AP2/ERF (1069), DOF(465), MYB (296), MIKS – MADS (233) and BBR-BBC (219). Other families possess less than 200 connections. Table 3 presents the top 10 TFs that have the most connections in each interaction network.

The question arises as to: how the families of TFs that have been identified affect sex determination; which processes they are involved in and with what interaction. The hormonal regulation plays a crucial role in the process of sex determination, as the genes primarily involved in this process are associated with ethylene synthesis, such as: *CsACS1*, *CsACS2*, *CsACS11* and they are linked with genetic loci *F*, *M*, and *A* respectively [21–24]. Expression and interaction among all three genes help in the development of female flower in cucumber. TFs act as a very important factor thatwhich can be either activate or repress the gene expression. Ethylene is the principal hormone that is responsible for the formation of specific organs and genes responsible for ethylene biosynthesis have a direct association with the development of female flowers [25,26]. The ethylene production in shoot apex primordia can readily modify the male to female flower ratio on the plant. It is known that sex in cucumber is linked to hormonal regulation and ethylene plays an important role in the cucumber. Of the identified TF families, ten are associated with the ethylene response in other plants and these are: AP2/ERF [27], MYB [28], NAC [29], bZIP [30], bHLH [31], WRKY [32], TCP [33], C2H2 [34], TALE [35] and MIKC_MADS [36]. Additionally, other hormones such as auxin and cytokinin exert a positive effect on female sex determination through interaction with ethylene biosynthesis and signaling pathways[37,38].The families connected with other hormones were also identified in this study: auxins – NAC [39], bHLH [40], LFY [41], cytokinin bHLH [40] and BBR-BPC [42,43]. The results of others studies demonstrated that gibberellins (GA) can have dual effects on sex expression

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in cucumber, inhibiting femaleness and inducing maleness and expression analysis has shown that CsACS1G transcription is promoted by auxins and inhibited by gibberellic acid [44,45]. According to the literature, there were five families: LFY [46], YABBY [47,48], MYB [49], BBR-BPC [42,43] and TALE [50] which were correlated with this hormone. AP2/ERF are identified as one of the largest groups of TFs in this study in all three comparisions. AP2/ERF family members induce ethylene signalling and flowering [51–53]. The *CsACS11* is one of the ethylene biosynthetic genes [54] and also thought to be a sex gene (*a*) in cucumber [22]. So far, it is not clear how the hormonal signalling pathways influence sex at the molecular level, so further detailed characteristics of the link between the regulatory TFs is needed. The formation of the complex flower architecture involves the MADS family described above. For the families identified, we found numerous TFs - DEGs links to flower development. Several studies reported role of MICKS-MADS [36,55,56], bHLH [57,58], bZIP [59], and NAC [60–62] in promoting or delaying flowering development. TALE family shows interaction with ethylene and cytokinin signalling [35]. Together with floral development, timimg of flowering is also crucial in plants for fruits and seed production. MYB [63], bZIP [64], bHLH [57,58] and WRKY [65] families are involved in regulation of flowering time, as desribed previously. Study in tomato, shows that bHLH acts with SFT or LFY and controls flowering time. It also influences ethylene biosynthesis genes, as the expression of ethylene biosynthesis genes is upregulated in the overexpression line of bHLH [66]. In the forming gynoecium of the Arabidopsis flower, other hormones such as auxin and cytokinin interact with bHLH [40]. BBR-BPC/GAGA has been described in Arabidopsis to regulate the phytohormonal signalling of cytokinins, brassinosteroids and ethylene [42,43]. The DOF TF has been reported to be involved in tissue differentiation, cell expansion, seed development, anther or pollen development and flowering in plants [67–69]. DOF has been also implicated in the formation of vascular tissue in reproductive organs [70]. The interaction between the bZIP member and the C2H2 member in melon inhibits the development of the carpel in male flowers [71,72]. It also represses the transcription of ethylene biosynthetic genes [73]. The present study reveals that the transcription factor WRKY is present solely in the FvH and FvM comparisons, while being absent in MvH. WRKY TF interacts with various flowering genes to regulate flowering timing in plants [65,74]. Another transcription factor, YABBY, is exclusively found in FvM comparison. The YABBY TF was desribed to play a crucial role in the development of anthers and pollen sacs in cucumber, Arabidopsis and rice [75–78]. The TFs from the YABBY family, interacts with MADS-box to control its expression during carpel development [79] In addition, only the LFY TF was identified in the FvM comparison, which is known to respond to auxin and regulate flowering initiation as presented in Arabidopsis [41,80].

A parallel and second approach to network analysis is to explore proteins (encoded by DEGs) to determine their reactivity with TFs regulators. In the compariosns of sex network such nodes were: for the FvH - DNA/RNA binding proteins, oxidoreductase proteins, for the FvM - pectinase, MADS box TFs and lipase proteins, and for the MvH - monooxygenase and triphosphate hydrolases.

Our analyses demonstrated the link between regulatory TFs and various developmental processes, including flower morphogenesis, flowering timing, and interactions with phytohormones. Gene expression governing specific functions involves the action of TFs. By conducting a detailed examination of interaction networks, we have identified regulatory TFs that have the potential to regulate a significant number of DEGs. These regulatory TFs act as central hubs in the network, and can influence a large portion of the nodes, thereby characterizing them as master regulators/hot links. The identification of these master regulators can serve as a valuable hub point for future investigations. By selectively focusing on these factors, their regulation or knockout, may be utilized to observe changes in DEGs within the context of sex comparison in cucumbers. This approach holds considerable potential for expanding our understanding of the complex regulatory networks underlying sex development in cucumber.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

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