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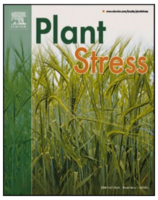

Comparative transcriptome analysis of *B. oleracea* L. var. italica and *B. macrocarpa* Guss. genotypes under drought stress: *de novo* vs reference genome assembly

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ABSTRACT

Drought stress influences plant growth and development affecting some physiological processes during the growing cycle. The improvement of the drought stress resistance of the plant will allow the reduction of the water requirements of the crops, representing the new frontier for agriculture, as consequence of the ongoing climatic changes. The individuation of genetic features useful for enhancing the reduction of water use and the plant drought resistance will be one of the key strategy for providing enough food for the foreseen increment of the global population. Nowadays, the cultivars used for modern agriculture show a narrow genetic diversity due to the domestication process carried out of each crop, resulting with a less adaptation to the environmental conditions affected by both antropic activities and the climatic change in act. Among the several grown species, *Brassica oleracea* L. ($n = 9$) crops are particularly vulnerable to the adverse effects of water deficiency. Transcriptomic analysis is a powerful tool that allows researchers to identify genes and pathways, that are activated or repressed in response to each specific stress, elucidating the complex regulatory networks that underlie the correspondent response. To individuate the differentially expressed genes we compared two drought-sensitive *B. oleracea* L. var. *botritis x italica* Sicilian landraces and two drought-tolerant *B. macrocarpa* Guss populations grown in ordinary irrigation regimes and in water deficit conditions. Their transcriptome was obtained by a RNAseq approach. The individuated sensible and tolerant drought stress genotypes showed significant difference for H_2O_2 content, leaf area and SPAD index (Soil Plant Analysis Development). For better identify crucial genes and pathways associated with drought stress response of both the sensible and tolerant genotypes a thorough evaluation of both *de novo* assembly and reference *B. oleracea* var. *italica* genome-based assembly was conducted. The comparison of the transcriptomes allowed to identify the crucial genes and pathways associated with tolerance to drought stress in *Brassica oleracea* L. crops. As main results, we individuated one gene coding transcription factor showing opposite behavior in the sensitive and tolerant signatures. The adoption of two transcriptome assembly methods provided a more comprehensive dataset, enabling an unbiased interpretation of the outcomes.

1. Introduction

Abiotic stress in plants encompasses a wide range of adverse environmental conditions that can negatively affect plant growth and development, thereby reducing crop yields (Imran et al., 2021; Yadav et al. 2020). They include drought, extreme temperatures (heat and cold), salinity, flooding, and heavy metal toxicity (Rajput et al. 2021; Gull, Ahmad Lone, and Ul Islam Wani 2019). These stressors disrupt essential physiological processes in plants, such as photosynthesis, respiration, and water uptake, resulting in cellular damage and metabolic imbalances (Saddhe, Manuka, and Penna 2021; Wu et al. 2022). Plant responses to abiotic stress involve a complex network of molecular

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and biochemical adaptations, including the activation of stress-responsive genes, the accumulation of protective compounds, and adjustments in water and nutrient uptake (Austen et al. 2019; Tiwari et al. 2020; Khan et al. 2020; Ghosh et al. 2022). The mitigation of the effects of abiotic stress plays a pivotal role in the development of innovative strategies to face the contemporary challenges in agriculture. These challenges include emerging environmental issues and the decreasing availability of arable land suitable for crop cultivation (Verma et al. 2020; Chaudhry and Sidhu 2021). Within this context, water deficiency represents an increasingly urgent worldwide issue, particularly in areas where agriculture is a major economic activity. The increasing global population exerts a mounting pressure on the agricultural sector for producing more food using less water (Rosa et al. 2020). The reduction of water uses in agriculture, the strategies for conserving water resources, improving agricultural productivity and enhancing drought stress resistance, are the new frontiers of agriculture, mostly in relation to the incoming climatic changes (Parkash and Singh 2020; Puglisi et al. 2019). Through the adoption of innovative water management techniques and the use of drought-resistant crop varieties, it is possible to drastically lower water consumption in agriculture, while maintaining or even increasing crop yields (Ricart et al. 2019). Nowadays, the crops used for modern agriculture show a limited genetic diversity due to the strong selection occurred during the domestication process, resulting with a less adaptation both to the environmental factors and to the new extreme climatic conditions (Prohens et al. 2017; Gaut et al. 2018). Among the several plant species damaged by drought, *Brassica oleracea* L. crops ($n = 9$) are particularly vulnerable to the adverse effects of water deficiency, with a substantial impact on both their yield and growth processes (Erken and Oztokat, n.d.; Kaluzewicz and Bosiacki 2018; Akram et al. 2024). *B. oleracea* crops, originating in the Mediterranean but domesticated across Mediterranean and Asiatic regions, are valued for their high antioxidant capacity. The bioactive compounds responsible for the above-mentioned properties, such as glucosinolates (GLSs) and polyphenols (PPs), were detected in high percentage in the crop wild relatives (CWRs) species - their utilization can be useful for introducing high nutraceutical traits to the cultivated species, producing lines with added nutritional values (Branca and Maggioni 2020). In *Brassica oleracea* complex species ($n = 9$), glucosinolates and polyphenols change in the different organs as a result of environmental stresses such as drought, as reported by different works (Ben Ammar et al. 2023, 2022). Varieties like broccoli, cauliflower, kale, cabbage, kohlrabi, and savoy cabbage have distinctive plant architectures owing to natural introgression of traits from CWRs. Brassica CWRs, in contrast to the cultivated crops, represent a source of resistance for abiotic and biotic stresses due to their several alleles lost during the domestication process, and they can be useful in breeding for transferring adaptive traits to the cultivated ones (Picchi et al. 2020). *B. macrocarpa* Guss is an endemic Brassica CWR which grows exclusively in the Mediterranean cliffs and in the rocky slopes of the Egadi Islands of Marettimo and Favignana (Sicily). It usually grows near the sea, and it is known for its high resilience to water deficiency conditions.

Transcriptomic analysis is a powerful tool that has revolutionized our understanding of plant stress responses and has the potential to drive the development of stress-tolerant crops, which is essential for sustainable agriculture and food security in the face of global environmental change. Transcriptomics allows researchers to identify genes and pathways that are activated or repressed in response to stresses and to elucidate the complex regulatory networks that underlie these responses (Imadi et al. 2015; Sicilia et al. 2019; Santoro et al. 2022; Sicilia et al. 2020). Moreover, transcriptomic analysis can also be used to identify molecular markers associated with stress tolerance and to develop crops with improved stress tolerance through conventional or traditional breeding programs (Agarwal et al. 2014). Various transcriptome assembly approaches exist, and it is worth noting two important methods: *de novo* assembly and assembly using a reference genome as a template. *De novo* assembly is an appropriate choice when studying non-model organisms or in the absence of a reference genome and it is a valuable aid with incomplete and poorly annotated genomes (Grabherr et al. 2011; Li and Dewey 2011; Góngora-Castillo and Buell 2013). Reference-based assembly, instead, provides greater accuracy and facilitates the identification of known genes, splice variants and regulatory elements, but suffers from lack of annotations (Liu et al. 2022). Several authors performed transcriptomic analysis in Brassica crops, using RNA sequencing techniques or developing a microarray chip, to detect the genes involved in different stress responses (Lee et al. 2008; Eom et al. 2018; Dai et al. 2022). They also assessed the drought stress response in B. oleracea. Previously, the involvement of stress-responsive glycosyltransferases in the mechanism of drought stress resistance was deepened in another work conducted by Rehman et al. (Rehman et al. 2018). They also compared the regulation patterns of these enzymes through transcriptomic analysis conducted on *Brassica oleracea, Brassica rapa*, and *Brassica napus*.

To individuate the differentially expressed genes we compared two drought-sensitive B. oleracea L. var. botritis x italica Sicilian landraces and two drought-tolerant B. macrocarpa Guss populations grown in ordinary irrigation regimes and in water deficit regimes. Their transcriptome was obtained by a RNAseq approach. The individuated sensible and tolerant drought stress genotypes showed significant difference for H_2O_2 content, leaf area and SPAD index (Soil Plant Analysis Development). For better identify crucial genes and pathways associated with drought stress response of both sensible and tolerant genotypes a thorough evaluation of both de novo assembly and reference B. oleracea var. italica genome-based assembly was conducted. The comparison of the transcriptomes allowed to identify the crucial genes and pathways associated with tolerance to water deficit conditions in *Brassica oleracea* L. crops. As main results, we individuated one gene coding transcription factor showing opposite behavior in the sensitive and tolerant signatures.

2. Materials and methods

2.1. Plant material and preliminary screening

The plant material consisted of 89 Brassica genotypes selected for preliminary screening from the active Brassica collection at the Department of Agriculture, Food and Environment (Di3A) of the University of Catania (UNICT). A detailed list of the accessions used can be found in Table S1. All accessions screened were part of the H2020 Breeding for Resilient, Efficient, Sustainable Organic Vegetable Production (BRESOV) project. The trial took place at the University of Catania during the summer of 2020, under organic farming conditions. Plantlets were sown in cellular trays with one seed per hole, using the organic soil substrate BRILL®semina bio (Geotec, Italy) for sowing. Sowing occurred in the second decade of May 2020. Plantlets received ample irrigation from the sowing date until the emission of the third true leaf. Subsequently, irrigation was withheld for a period of five days in June 2020, except for the control blocks. The comprehensive analysis involved six biological replicates for each accession, conducted under both irrigated and stressed conditions. This replicates have been growth in an “open greenhouse” in July 2020 with an average temperature between 24.5 and 27.5° (see Figure S1). Plantlets were assessed for various morphometric traits, including the total number of leaves, chlorotic leaves, and dry leaves. Chlorophyll content was measured using the SPAD-502 device (Konica Minolta Optics, Japan). Additionally, plants were evaluated for their resilience to drought stress using a numerical scoring system, with a score of 0 indicating the most sensitive plantlets, 1 for intermediate tolerance, and 2 for highly tolerant ones.

2.2. Data analysis for preliminary screening

The genotypes selection for subsequent analysis was conducted through morphological observations, assessing adaptation to water deficit conditions, and by analyzing the variation in total leaf number and SPAD index values between stressed and irrigated control conditions. The analysis was performed using the following formula:

$$\Delta \mathrm{L} = (\mathrm{LsLc} - 1 -)100$$

where ΔL indicates the variation between the two conditions; Ls represents the number of leaves registered in stress condition; Lc refers to the number of leaves registered in control condition.

On the other hand, the same formula was applied for the SPAD value:

$$\Delta \mathrm{S} \ = \ (\mathrm{Ss}\ Sc - 1)\ 100$$

where ΔS indicates the variation between the two conditions; Ss represents the SPAD index recorded in stress condition; *Sc* is the SPAD index recorded in control condition. Finally, ΔL and ΔS were statistically analyzed by SPSS software version 27 (IBM, Armonk, USA) performing principal component analysis.

2.3. Genotypes selection and stress imposition

Four genotypes were selected among the initial 89 accessions based on their different response to stress imposition and are listed in Table S2. The stressed plantlets were irrigated until the total soil saturation, and they were recovered for 18 days irrigating the alveolar trays daily. After that, they were transplanted from the trays and placed into 20 cm diameter pots having the hydraulic capacity of 4.5 L for the further stress application, which was applied ten days after the transplanting. The tested genotypes underwent assessment under two distinct irrigation regimes (IR). Genotypes receiving regular irrigation constituted the control group, denoted as the Irrigated (IRR) condition. Conversely, those subjected to water stress were classified under the non-irrigated (NIR) condition. The stress was applied for seven days, while the control genotypes were well irrigated, applying 25 cl of water per pot, per day. At the seventh day, plants were characterized for their total, chlorotic and dry leaves in addition to their leaf chlorophyll index obtained by SPAD (Konica Minolta Optics, Japan) and to the morphometric traits, such as the leaf lamina area (cm^2), the leaf length (cm) and the leaf width (cm). Fresh samples were collected and stored at −80 °C for further analysis.

2.4. Chemical analysis

To assess the drought stress tolerance or sensitivity, the leaves of selected plants were analyzed for their Malonaldehyde (MDA) and hydrogen peroxide (H_2O_2) amount, which represents metabolites related to the oxidative stress response. The amount of MDA was calculated following the protocol provided by Lopez-Hidalgo *et al.* (López-Hidalgo et al. 2021), while the H_2O_2 was calculated by the protocol of Velikova *et al.* (Velikova, Yordanov, and Edreva 2000).

2.5. Transcriptomic analysis

2.5.1. RNA extraction

The leaves, kept frozen by continuously liquid nitrogen adding, were ground using precooled mortar and pestle. Total RNA was isolated by using the extraction protocol Spectrum Plant Total RNA kit (Sigma-aldrich®, Saint Louis, MO, USA). RNA degradation and contamination were monitored by electrophoresis with 1 % agarose gel. RNA purity and concentration were assayed using the NanoDrop spectrophotometer (TermoFisher Scientifc, Waltham, MA, USA) (Russo et al. 2021). Before being sequenced, the RNA samples were subjected to quality parameter evaluation. RNA integrity was assessed using the Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA)(Sicilia et al. 2019).

2.5.2. Library preparation

One μg of RNA was used as input material for library preparations (twentyfour libraries: two irrigation conditions x four accessions x three individuals). Sequencing libraries were generated using NEBNext ®Ultra ™ RNA Library Prep Kit for Illumina® (New England Biolabs, Ipswich, MA, USA) following manufacturer's recommendations (Russo et al. 2021). Briefy, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Bufer (5X). First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H) as synthesizing enzyme (Russo et al. 2021). Second strand cDNA synthesis was subsequently performed using RNase H to insert breaks into the RNA molecule and DNA Polymerase I as synthesizing enzyme. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3′ ends of DNA fragments, NEBNext Adaptor with hairpin loop structure were ligated to prepare for hybridization. To select cDNA fragments of preferentially 150~200 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, MA, USA). Then 3 μl USER Enzyme by NEB were used with size-selected, adaptor-ligated cDNA at 37 °C for 15 min followed by 5 min at 95 °C before PCR. PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. Finally, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system (Sicilia et al. 2019).

2.5.3. Clustering and next generation RNA sequencing

Cluster generation and sequencing were performed by Novogene (UK) company Limited (25 Cambridge Park, Milton Road, Cambridge, CB4 OFW, United Kingdom). The clustering of the index-coded samples was performed on a cBot Cluster Generation System using a PE Cluster kit cBot-HS (Illumina). After cluster generation, the library preparations were sequenced on Illumina HiSeq2000 platform to generate paired-end reads whose size was paired-end 2 × 150 bp reads (Santoro et al. 2022; Russo et al. 2021)· Raw reads in fastq format were firstly processed through in-house perl scripts. In this step, clean data were obtained by removing reads containing adapters, reads containing poly-N and low quality reads. At the same time, Q20, Q30, GC-content and sequence duplication level of the clean data were calculated. All the downstream analyses were based on clean data with high quality (Table S3).

2.5.4. De-novo assembly, functional annotation and enrichment and differential expression analysis

De novo transcriptome assembly has been performed by making use of Trinity software (2.6.6 version, min_Kmer_Cov = 3 and min_glue = 4 (Grabherr et al. 2011)). Hierarchical Clustering was carried out by Corset (4.6 version) to remove redundancy (parameter -m 10), so that the longest transcript of each cluster has been selected as Unigene (Davidson and Oshlack 2014). The assembly assessment and gene prediction have been performed by Benchmarking Universal Single-Copy Orthologous (BUSCO software, 3.0.2 version) (Simão et al. 2015), whereas the gene functional annotation was obtained by exploiting seven different databases: National centre for Biotechnology Information (NCBI), non-redundant protein sequences (Nr, Diamond software, 0.8.22 version, e-value threshold 1e-5) (Buchfink, Xie, and Huson 2015), NCBI non-redundant nucleotide sequences (Nt, NCBI blast software, 2.9.0 version, e-value threshold 1e-5), Protein family (Pfam, hmmscan software, HMMER 3.1 version, e-value threshold 0.01) (Buchfink, Xie, and Huson 2015; Finn, Clements, and Eddy 2011), Cluster of Orthologous Groups of Proteins (KOG/COG, Diamond software, 0.8.22 version, e-value threshold 1e-5), Swiss-Prot (Diamond software, 0.8.22 version, e-value threshold 1e-5) (Buchfink, Xie, and Huson 2015), Kyoto Encyclopedia of Genes and Genome (KEGG, Diamond and KAAS software, 0.8.22 version, e-value threshold 1e-5) (Buchfink, Xie, and Huson 2015; Finn, Clements, and Eddy 2011; Moriya et al. 2007) and Gene Ontology

(GO, blast2GO software, b2g4pipe_v2.5 version, e-value threshold 1e-6). Gene expression level was estimated by RSEM software (1.2.28 version) by mapping back each clean read onto assembled transcriptome and read counts for each gene were then obtained from the mapping results. Furthermore, the read counts of each gene have been used as input data for DESeq2 (1.26 version, padj$\leq$0.05), to obtain differentially expressed genes (DEGs) (Santoro et al. 2022). An adjusted p-value cutoff of 0.05 and a log2fold change (Log2FC) threshold of 1 was adopted to filter the significantly up- and down-regulated genes.

2.5.5. *Reference assembly*

We developed a custom pipeline for RNAseq analysis of plant reference genomes. Initially, Trimmomatic (v. 0.39) (Bolger, Lohse, and Usadel 2014) was utilized to filter out poor-quality reads and adapters. Subsequently, STAR (Dobin et al. 2013) was employed as a gene-level aligner, which also provided raw counts without the need for an intermediary program to analyze the BAM files. STAR indexes were constructed using Brassica Oleracea TO1000 as the reference genome from NCBI. Differential expression analysis was conducted using the edgeR (Cheng et al. 2022) and Limma (Ritchie et al. 2015) packages of R (v. 4.2.2). Genes were considered significantly up- or down- regulated only if the adjusted p-value was < 0.05. Each gene was annotated using the UniprotR package (v. 2.2.2) (UniProt Consortium and The UniProt Consortium 2018)'(Soudy et al. 2020). In cases where annotation was not available in Uniprot, BlastKoala (Kanehisa, Sato, and Morishima 2016) was employed to infer gene function by identifying high similarity percentages with genes from other plants. Enrichment analysis was performed using clusterProfiler (Yu et al., 2012) (v. 4.6.0), AnnotationHub (Akalin, 2020) (v. 3.6.0), Biomartr (Drost and Paszkowski 2017) (v. 1.0.2), and AnnotationDbi (Gentleman 2008) (v. 1.60.0).

2.6. *Bioinformatic analysis*

For every analyzed cultivar three stressed and three control replicates have been examined. First of all, statistical analysis was performed using the Shapiro-Wilk test to assess the normality of the sample distributions. The Levene test was applied to evaluate the homogeneity of variances across groups. Based on these results, we proceeded with an ANOVA or a Kruskal-Wallis test for comparing group means. Pairwise comparisons were conducted using the Wilcoxon test for non-parametric data and the *t*-test for parametric data, with corresponding p-values calculated for each test. Principal Component Analysis (PCA) was conducted on phenotypical and chemical characteristics by employing the R package Factoextra ("Extract and Visualize the Results of Multivariate Data Analyses" n.d.) (v.1.0.6).

2.7. *Investigated signatures*

Three signatures were obtained by using the Differential Expression Analysis (DEA) results to emphasize the role of Differential Expressed Genes (DEGs) in the drought stress intersecting specific plants' DEGs for both reference and de-novo analysis. In particular, the tolerance or the sensitivity of the genotypes studied was considered to obtain sensitive signature, tolerant signature and intersection signature (Fig. 1): (i) the sensitive signature was created by intersecting the DEGs inferred from BS (Sensitive) cultivar (DR vs CK) with those obtained from BM (Medium Sensitive) cultivar (DR vs CK), (ii) the same intersection has been done to create the tolerant signature using MM (Medium Tolerant) and MF (Tolerant) cultivars, (iii) finally, a joint signature which compares each tolerant cultivar versus the sensitive one both for stressed and control plants was obtained. Then, the results of tolerant plants were subtracted from the results of their respective stressed plants. Finally the four lists of genes have been intersected and the common genes are retained as signatures.

3. Results

3.1. *Selection of the subset of sensitive and tolerant accession*

The results concerning the preliminary screening cycle performed for the selection of the 2 most sensitive and tolerant genotypes, are summarized in Fig. 2 obtained considering the parameters listed in Table S4. The selected genotypes were positioned at opposite ends of the PCA plot. This positioning indicated significant differences, with high ΔL and ΔS values observed for the tolerant B. macrocarpa Guss. accessions, while the two B. oleracea var. italica landraces exhibited lower values of ΔL and ΔS (Fig. 2). In the PCA plot, the accessions indicated as tolerant and selected are BM3 and BM4 (crop codes MM and MF). Conversely, the accessions selected as sensitive and indicated in the PCA plot, were BR15 and BR13 (crop codes BM and BS). In Table S2 are reported information about crop code, species name and origin

3.2. *Phenotypic and chemical analysis of the selected genotypes*

Statistical analysis of phenotypic and chemical traits in the selected genotypes confirmed the visual evaluation hypothesis regarding the tolerance types of the cultivars. While the overall leaf count remained consistent between sensitive and tolerant NIR plants, a notable observation was the distinct wavy pattern in leaf shape (Fig. 3). Specifically, among the cultivars listed in Table 1, we observed a significantly greater leaf width in NIR BS and BM compared to MF and MM. However, this pattern was not observed under irrigated conditions for the same plants. A similar trend was observed for leaf lamina length, which was significantly smaller in NIR-tolerant plants compared to sensitive ones.

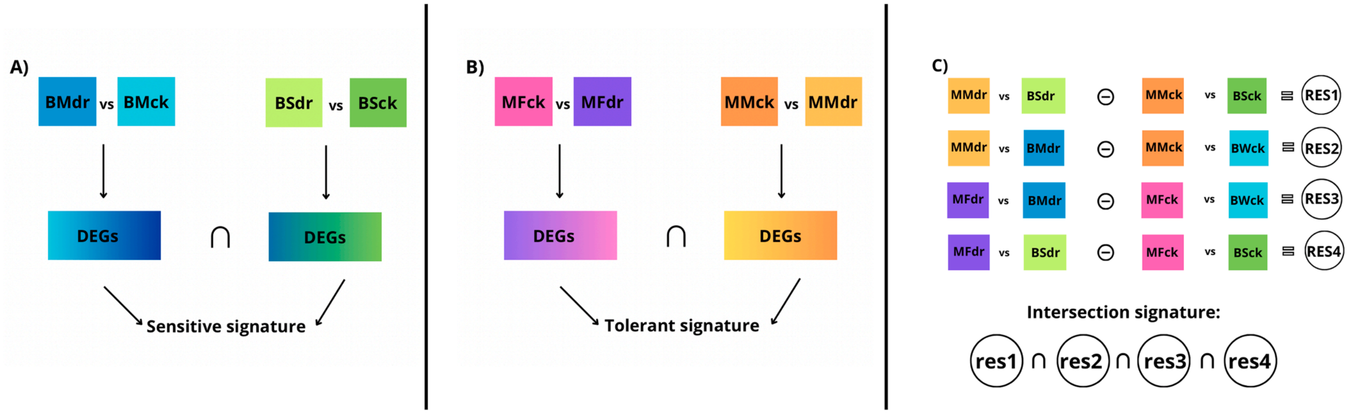

Fig. 1. Descriptive diagram which explains how sensitive signature (A), tolerant signature (B) and intersection signature (C) have been obtained.

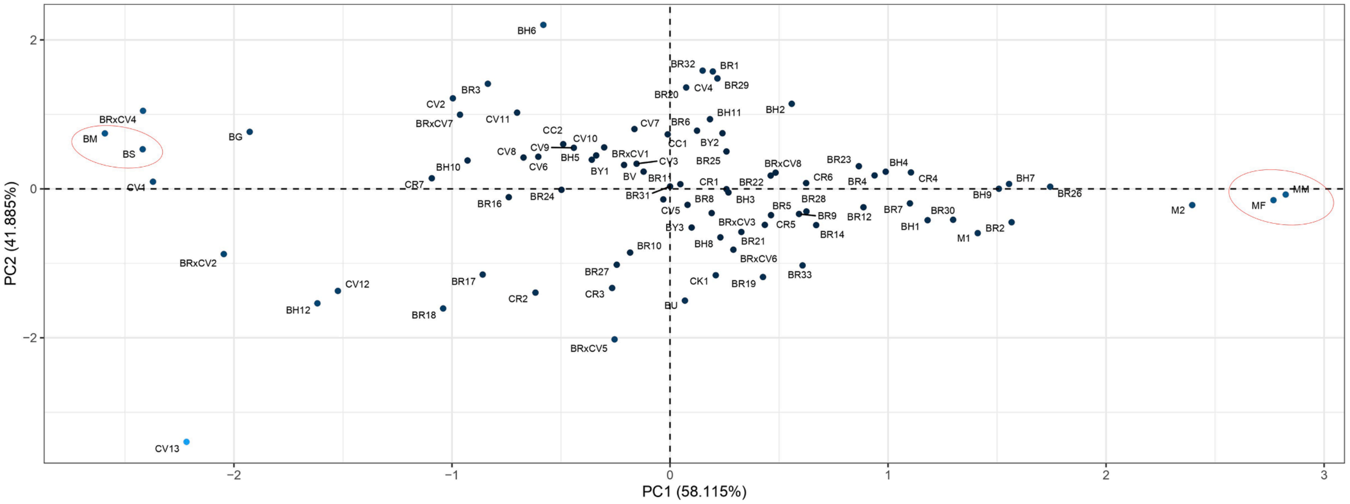

Fig. 2. PCA plot of the 86 accessions evaluated in the preliminary screening cycle. Red circles indicate the tolerant and sensitive accessions selected (right and left circle, respectively).

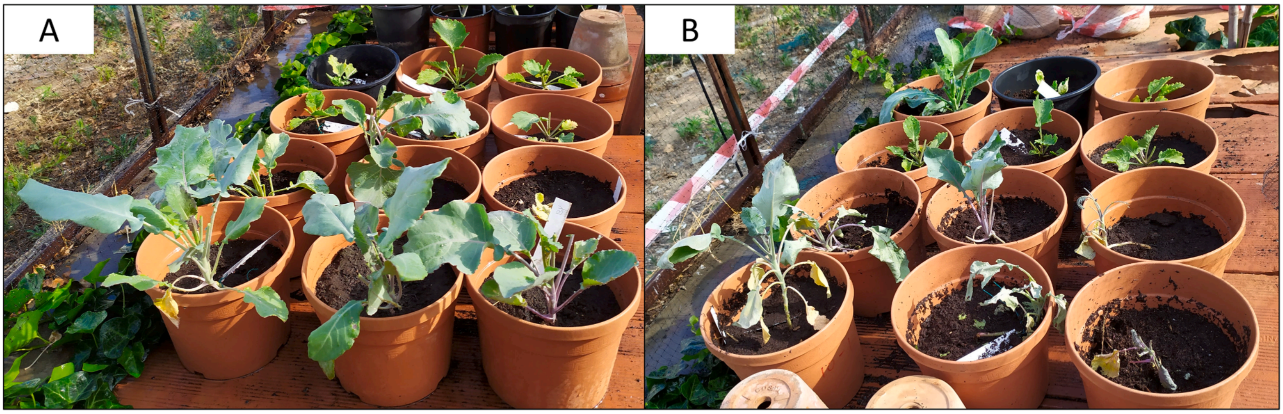

Fig. 3. Control (A) and stressed (B) accession analyzed for the water deficit trial.

Notably, the differences were significant between MM and BS (p-value < 0.001), and among MF and BS, and MF and BM (p-value < 0.05), but these differences were not detected under irrigated conditions. In contrast, within the irrigated genotype set, the SPAD index displayed no significant variations. However, among the NIR accessions, the SPAD index varied significantly between plants identified as tolerant and those identified as sensitive (Table 1). In particular, the SPAD index of MM was significantly higher than that of BM and BS, with a p-value < 0.01. Regarding hydrogen peroxide content (H_2O_2), it's noteworthy that the most sensitive plant (BS) under irrigated conditions exhibited a significantly higher level of this metabolite compared to the moderately tolerant IRR plant (MM). As shown in Table 2, leaf width, leaf area, and leaf lamina length increased significantly in NIR-sensitive plants compared to tolerant ones. The only parameter that was significantly different between sensitive and tolerant plants even under irrigated conditions was leaf area, which was already higher in sensitive plants. Concerning physiological traits, we observed significant changes in H_2O_2 and SPAD between sensitive and tolerant plants under NIR conditions but these traits remained stable under irrigated conditions. We also analyzed MDA, but it remained unaltered between the two different irrigation conditions.

By taking into account both chemical and phenotypic characteristics we compared the different cultivars using a PCA. As it can be seen in Fig. 4 using these traits it has been possible to cluster the plants with their response characteristics to the drought stress. Moreover, from Fig. 4A it is possible to notice how the tolerant plants cluster close to the control ones. On the other hand, in Fig. 4B we can observe that the variables "Leaf Area", "H_2O_2" and "Leaf Lamina Length" are those contributing more to this plants' classification.

3.4. Transcriptomic analysis

In our molecular analysis, we conducted a transcriptomic study to identify Differentially Expressed Genes (DEGs) between pairs of plants subjected to drought stress and those without. Table S3 displays the percentage of reads with q30 ranging from 85 % to 92 %, indicating good sequencing quality. Furthermore, BUSCO analysis resulted in about 70 % of complete and single copy Unigenes, indicating a good reliability and quality of the *de novo* transcriptome (Figure S2). Comparing stressed plants of each accession with their respective controls, we observed a higher number of DEGs in the sensitive accessions BM and BS (912 and 1147, respectively) compared to the tolerant accessions MF and MM in the reference genome analysis (514 and 383, respectively). Interestingly, this trend reverses when considering the De novo analysis, where the highest number of DEGs was recorded in the tolerant accessions MF and MM (3184 and 4446, respectively) (Table 3).

To identify signatures of tolerance and sensitivity among cultivars, we examined the genes common to each group (Venn Diagrams can be found in supplementary material Figure S3). We found only 9 genes common among all four cultivars in the reference genome analysis and

Table 1
Variation of the morphometric and chemical parameters in relation to the water deficit application. Particularly, the traits reported were the number of the total, chlorotic and dry leaves (n), SPAD index, leaf area (cm^2), lead lamina length and width (cm), as well the H_2O_2 (%) and MDA (ng/mg). ***, **, * indicate p value ≤ 0.001, 0.01, 0.05, respectively. The values in the table represent the mean and the standard deviation values of three biological replicates for each accession.

	NIR									IRR								
	Total Leaves (n)	Chlorotic Leaves (n)	Dry Leaves (g)	SPAD	Leaf Area (cm^2)	Leaf Lamina Lenght (cm)	Leaf width (cm)	H_2O_2 (%)	MDA (ng/mg)	Total Leaves (n)	Chlorotic Leaves (n)	Dry Leaves (g)	SPAD	Leaf Area (cm^2)	Leaf Lamina Lenght (cm)	Leaf width (cm)	H_2O_2 (%)	MDA (ng/mg)
MF	8 (1)	3 (1)	0.33 (0.58)	42.9 (5.11)	40.03 (1.92)	7.73 (0.62)	5.24 (0.26)	0.23 (0.14)	1.53 (0.6)	8 (1)	0 (0)	0.33 (0.58)	47.87 (4.07)	38.83 (2.16)	7.68 (0.64)	5.2 (0.22)	0.35 (0.32)	2.03 (0.67)
MM	8 (1)	2 (0)	0.33 (0.58)	53.9 (3.17)	40.9 (0.61)	7.88 (0.18)	5.47 (0.36)	0.2 (0.12)	1.17 (0.39)	9.67 (1.15)	0 (0)	0 (0)	48.4 (9.08)	38.33 (1.96)	7.82 (0.17)	5.72 (0.46)	0.14 (0.06)	2.37 (1.32)
BM	7 (1)	3.67 (0.58)	1.33 (0.58)	35.5 (3.54)	58.4 (2.65)	9.38 (0.69)	8.06 (0.73)	0.92 (0.51)	1.82 (0.71)	9 (0)	0.5 (0.7)	0 (0)	53.4 (0.99)	40.7 (0.71)	8.28 (0.1)	5.7 (0.34)	0.35 (0.13)	5.1 (1.16)
BS	8.33 (0.58)	3.33 (0.58)	0.3 (0.58)	32.4 (2)	52.5 (3.67)	9.7 (0.19)	7.18 (0.26)	1.28 (0.33)	1.59 (0.71)	9.67 (0.58)	0.33 (0.58)	0.33 (0.58)	48.1 (7.04)	46.9 (5.47)	8.19 (0.35)	6.15 (0.99)	0.53 (0.15)	2.14 (0.55)
MM vs MF	ns	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
MM vs BM	ns	ns	ns	**	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	*	ns	ns	ns
MM vs BS	ns	ns	ns	**	ns	***	**	*	ns	ns	ns	ns	ns	ns	ns	ns	*	ns
MF vs BM	ns	ns	ns	ns	ns	*	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
MF vs BS	ns	ns	ns	ns	ns	*	***	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
BM vs BS	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Table 2

Variation of the morphometric and chemical parameters in relation to not irrigated (NIR) tolerant and sensitive plants, as well for irrigated (IRR) tolerant and sensitive plants. ****,***, **, * indicate p value ≤ 0.0001, 0.001, 0.01, 0.05, respectively. The values in the table represent the mean and standard deviation values of three biological replicates for each accession of each condition.

Samples	Total Leaves (n)	Chlorotic Leaves (n)	Dry Leaves (g)	SPAD	Leaf Area (cm^2)	Leaf Lamina Length (cm)	Leaf Width (cm)	H_2O_2 (%)	MDA (ng/mg)
Tolerant stressed	8	2.5	0.33	48.4	40.47	7.81	5.35	0.21	1.35
(MFdr and MMdr)	(0.89)	(0.84)	(0.52)	(7.14)	(1.36)	(0.42)	(0.31)	(0.12)	(0.5)
Sensitive stressed	7.67	3.5	0.83	33.9	55.4	9.54	7.62	1.1	1.7
(BMdr and BSdr)	(1.03)	(0.55)	(0.75)	(3.1)	(4.31)	(0.48)	(0.69)	(0.43)	(0.65)
Tolerant control(	8.83	0	0.17	48.1	38.6	7.75	5.46	0.24	2.2
MFck and MMck)	(1.33)	(0)	(0.41)	(6.3)	(1.87)	(0.43)	(0.43)	(0.24)	(0.96)
Sensitive control(	9.4	0.4	0.2	50.2	44.4	8.23	5.96	0.46	3.33
BMck and BSck)	(0.55)	(0.55)	(0.45)	(5.78)	(5.14)	(0.25)	(0.76)	(0.15)	(1.77)
Sensitive stressed vs Tolerant Stressed	ns	ns	ns	**	**	****	**	**	ns
Sensitive control vs Tolerant control	ns	ns	ns	ns	**	ns	ns	ns	ns

133 genes in the De novo analysis. The sensitive signature comprised 320 genes in the reference analysis and 545 in the De novo analysis, while the tolerant signature contained 27 genes in the reference analysis and 498 in the De novo analysis. Additionally, the intersection signature included 356 genes in the reference genome analysis and 658 in the De novo analysis (Supplementary Files S1-S6). Among the investigated genes, Bo9g041010 (homologous to Arabidopsis thaliana Transcription factor bHLH112, SwissProt Q94JL3) was common to both tolerant and sensitive cultivars, displaying a clear expression trend (See Supplementary File S3 and Fig. 5). Specifically, it was upregulated in tolerant cultivars and downregulated in sensitive ones. However, this differential expression was only statistically significant for BS and MF, as indicated by the adjusted p-value.

3.5. *Gene ontology analysis of tolerant and sensitive signature*

Fig. 6 provides a comprehensive overview of the Gene Ontology (GO) results corresponding to each gene within the respective signatures. This figure shows GO annotations filtered to include only those terms containing at least 5 genes, while the complete list can be accessed in supplementary files. Our GO analysis encompasses both the reference genome signatures and the de novo signatures, with a focus on Biological Process (BP). The analysis conducted on the sensitive signature resulted from the reference analysis returned 36 genes annotated in different GO terms. Fig. 6A shows the main annotated Biological Processes in the sensitive signature. They include Regulation of transcription, DNA-templated (GO:0006355) with Bo01141s010 involved also in auxin-activated signaling pathway (GO:0009734); Response to abscisic acid (GO:0009737) with the genes Bo1g117540, Bo3g001410, Bo3g132570, Bo3g134720, Bo4g014570, Bo8g091960, Bo9g004450 (see Table 4); methylation (GO:0032259); pectin catabolic process (GO:0045490); carbohydrate metabolic process (GO:0005975); cell wall modification (GO:0042545) and response to salt stress (GO:0009651). In our De novo analysis related to the sensitive signature in the context of Gene Ontology (GO), we identified 37 functionally annotated genes, with the majority (over 30) involved in the regulation of transcription, DNAtemplated (GO:0006355). Additionally, processes such as protein phosphorylation (GO:0006468) and sucrose metabolism process (GO:0005985) were strongly involved in the response of sensitive accessions to drought stress (Fig. 6D). These findings underscore the complexity and diversity of biological processes involved in the sensitive signature identified in our De Novo analysis, highlighting both known pathways and areas warranting further investigation. In Fig. 6B, the functional gene annotations related to Biological Processes (BP) within the tolerant signature of the reference analysis highlights crucial functions characterizing tolerant plants, such as the auxin-activated signaling pathway (GO:0009738) (genes Bo1g016790 and Bo2g134160) and the abscisic acid-activated signaling pathway (GO:0009734) (genes Bo7g075740 and Bo4g190030). Turning to the de novo analysis presented in Fig. 6E, a notable gene count is observed in the regulation of transcription, DNA-templated (GO:0006355) followed by the protein phosphorylation (GO:0006468) and transmembrane transport (GO:0055085). In Fig. 6C, the intersection signature reveals four significant terms in Biological Processes (BP), with protein phosphorylation (GO:0006468) having the highest gene count of 15, followed by protein ubiquitination (GO:0016567) and regulation of DNA-templated transcription (GO:0006355). Shifting to the de novo analysis signature in Fig. 6F, the BP terms exhibit a different order of the terms compared to the reference genome analysis. The regulation of DNA-templated transcription (GO:0006355) is the richest (40 genes), followed by protein phosphorylation (GO:0006468)and transmembrane transport (GO:0055085).

3.6. *Pathways analysis of tolerant and sensitive signature*

Pathway analysis using KEGG was conducted for both the reference genome and de novo analyses, as illustrated from Figs. 7 to 9. In Fig. 7A, six KEGG pathways have been found perturbed in the reference genome analysis with the sensitive signature. Notable among these pathways: (i) ABC transporters, involving genes Bo4g186480, Bo2g047420, and Bog9g008680; (ii) Motor protein, with gene Bo5g126420, and gene Bo3g116270, which is also linked to (iii) Phagosome, involving genes Bo4g023960, Bo1g016970, and Bo1g006800. (iv) Propanoate metabolism, featuring genes Bo9g022510 and Bo4g125890, which is further connected to (v) Biosynthesis of unsaturated fatty acid, and (vi) Fatty acid metabolism, which also includes Bo7g026690 (Fig. 7A). In the de novo analysis (Fig. 7B), five perturbed pathways are observed. Among them, Fatty acid degradation, previously identified in the reference genome, is noteworthy. Additionally, the de novo analysis highlights other perturbed pathways, including: (i) Biosynthesis of secondary metabolites, (ii) Plant hormone signal transduction, and (iii) Starch and sucrose metabolism. In Table 5 are listed genes belonging to these pathways involved in stress response. In particular genes encoding for probable protein phosphatase 2C 78 and Indole-3-acetic acid-amido synthetase GH3.6 have been found up-regulated, genes encoding for Cytochrome P450, Abscisic acid receptor PYR1, Auxin transporter-like protein 3, Auxin-responsive protein SAUR50 resulted down-regulated in the sensitive signature (Table 5), suggesting their involvement in the sensitive response to drought stress. In Fig. 8A, the reference genome analysis of tolerant signature shows only one significant pathway: the MAPK signaling pathways in which are involved two significant genes: Bo4g190030 and Bo7g075740 (Table 4). In de novo analysis shown in

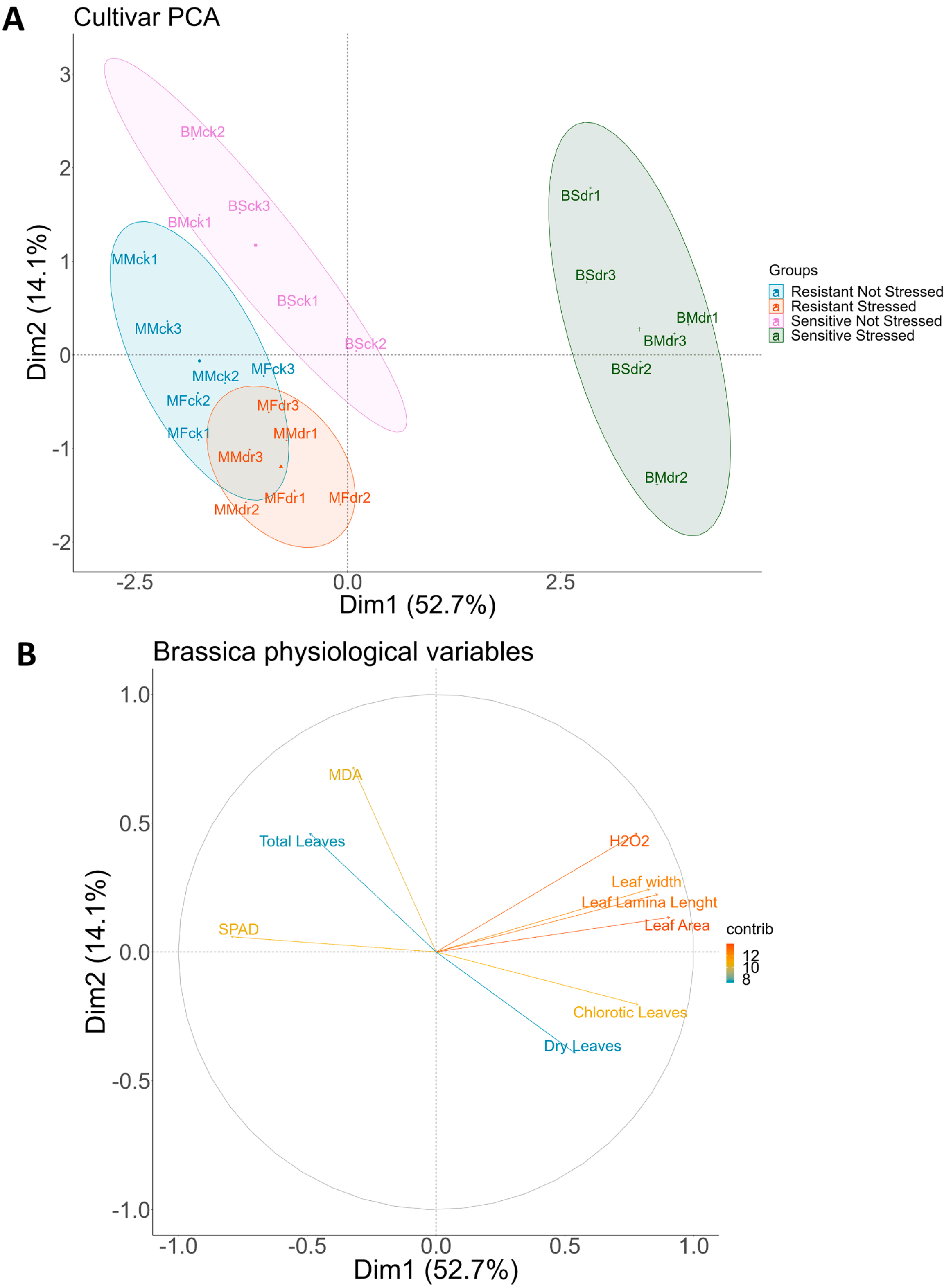

Fig. 4. PCA on the basis of the cultivars characteristics. A) distribution of accessions in relation to drought stress; B) Contributions of the chemical and phenotypic characteristics.

Fig. 8B we can find several significant pathways associated with tolerant signature. Among them it is notable to mention the metabolic pathways and the biosynthesis of secondary metabolites, including genes such as ABSCISIC ACID-INSENSITIVE 5-like protein 6 and Protein phosphatase 2C (up-regulated), Magnesium dechelatase SGRL, chloroplastic, Transketolase-1, chloroplastic and Auxin-responsive protein IAA28 (down-regulated) (Table 5). Finally, Pathways network of intersection signature with reference genome, in Fig. 9A, shows ten perturbed pathways. The pathways (i) Arginine biosynthesis, (ii) Tyrosine metabolism, (iii) Phenylalanine metabolism, (iv) Alanine, aspartate and

Table 3
Number of Differentially Expressed Genes (DEGs) in control (CK) and stressed (DR) plants of the four accession analysed.

Accessions	Degree of Resistance	DEGs in reference genome analysis (n)	DEGs in de novo analysis (n)
MF	Tolerant	514	3184
MM	Medium Tolerant	383	4446
BM	Medium Sensitive	912	1591
BS	sensitive	1147	2890

glutamate metabolism, (v) Butanoate metabolism, (vi) Pantothenate and CoA biosynthesis, (vii) Valine, leucine and isoleucine biosynthesis, (viii) 2-Oxocarboxylic acid metabolism are all concatenated trough at least one gene. While (ix) Motor Protein and (x) Phagosome are concatenated by the gene Bo3g116270. In the de novo analysis, Fig. 9B, the Kegg Orthology highlights the biosynthesis of secondary metabolites with about 50 gene. This pathway is followed by Plant hormone signal transduction, Biosynthesis of cofactors, mRNA surveillance pathway and Starch and Sucrose metabolism. The main genes involved in these pathways are Cytochrome P450 83A1 (up-regulated) and 9-cis-epoxy-carotenoid dioxygenase NCED3 chloroplastic (down-regulated) (Table 5).

4. Discussion

Abiotic stresses are widely considered as severe environmental factors that significantly impairs crop production worldwide. Plants generally experience a wide range of abiotic stressors, including high levels of salt (salinity), extreme temperatures (chilling, freezing or heat) and insufficient water availability (drought or dehydration). These stressors collectively represent the primary drivers behind the substantial reduction of the yield of crops (Waadt et al. 2022). Overall, the susceptibility or tolerance of plants to these stresses emerges because of the intricate interplay of multiple stress-responsive genes. Consequently, these genes concurrently interact through the cross-talk with other components within the stress signal transduction pathways (Tuteja 2007). Among the stressors cited above, water deficiency represents an increasingly urgent worldwide issue, particularly in areas where agriculture is a major economic activity. The reduction of water used in agriculture, the strategies for conserving water resources, improving agricultural productivity and enhancing drought stress resistance, are the new frontiers of agriculture, mostly in relation to the incoming climatic changes. Among the several plant species damaged by drought, *Brassica oleracea* L. crops ($n = 9$ chromosomes), valued for their high antioxidant capacity conferred by bioactive compounds such as glucosinolates (GLSs) and polyphenols (PPs), are particularly vulnerable to the adverse effects of water deficiency, with a substantial impact on both their yield and growth processes. For these reasons, our research has been centered on conducting transcriptomic analysis on four accessions within the *Brassica oleracea* complex species selection showing a different response to water deficit imposition (tolerance and sensitivity). The transcriptomic response of the tolerant and sensitive genotypes was thus compared with the aim of identifying specific genes and pathways associated with the plant's response to drought stress. A noteworthy aspect of our research regards the comprehensive comparison of two distinct bioinformatics approaches for transcriptome assembly: reference and de novo. As it emerged by phenotypic analysis, *B. macrocarpa* accessions exhibited a notable drought stress tolerance. This assertion finds reinforcement in its native habitat, which is characterized by rocky slopes along the coast of Egadi's Island, in which this particular genetic resource thrives an endemism. Conversely, the identified sensitive broccoli landraces belong to the "ciurietto" group, a category encompassing cultivars exhibiting intermediate traits between broccoli and cauliflowers. It is widely recognized that these cultivars were selected in Sicilian local gardens due to their astonishing organoleptic properties and they were not selected for their resilience traits (Ferdinando Branca et al. 2017). In our analysis of morphological traits, we did not detect substantial variations in the total and chlorotic leaf counts in both NIR and IRR conditions between sensitive and tolerant plants. The different response to water deficit imposition between sensitive and tolerant plants can be observed when considering SPAD, leaf area, leaf lamina length and leaf width.

As concerning the analysis of the two metabolites significantly involved in the oxidative stress (MDA and H_2O_2), no differences in MDA

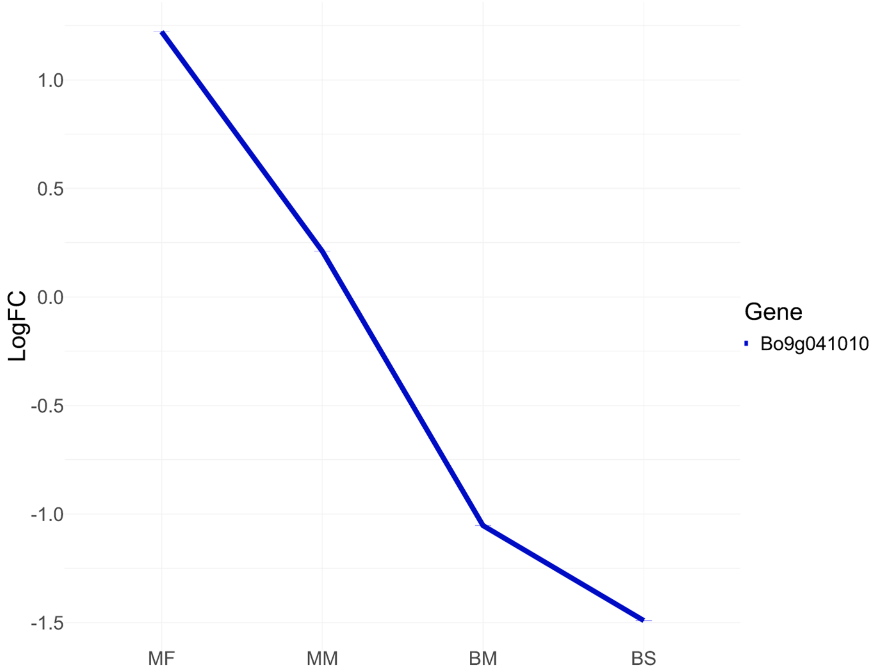

Fig. 5. Line plot depicts the behavior of gene Bo9g041010 in the whole group of cultivars. The gene was upregulated in tolerant plants (MF and MM) and downregulated in sensitive ones (BM and BS).

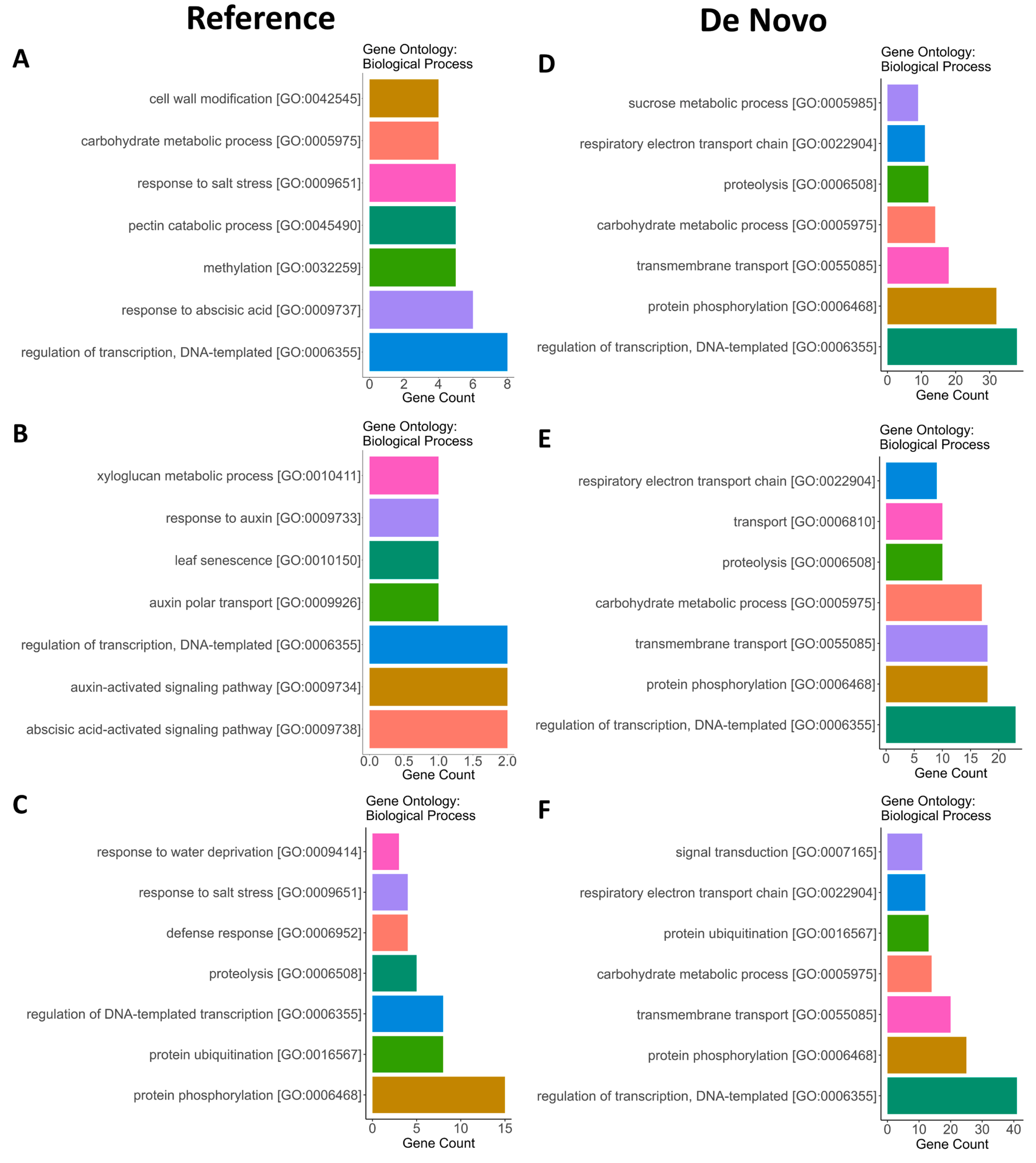

Fig. 6. GO Barplot of Biological Process (BP) of both the reference genome (A,B,C) and de novo analysis (D,E,F) signatures, describing (A,D) sensitive signature, (B,E) tolerant signature, (C,F) intersection signature.

content were measured in both IR when comparing sensitive and tolerant plants. Meanwhile, in NIR condition the sensitive plants showed a significantly higher value of H_2O_2 with respect to the tolerant plants, indicating that a high level of secondary oxidative stress triggered by water stress has been achieved in sensitive plants. In fact under normal physiological activities, plants produce reactive oxygen species (ROS), such as superoxide anion radicals (O2−), singlet oxygen (O2), hydroxyl radicals (·OH) and hydrogen peroxide (H_2O_2), as signal transmitters to regulate gene and protein expression in plant cells, and the production and elimination of ROS are always in a state of dynamic equilibrium (Waszczak, Carmody, and Kangasjärvi 2018). When the plant is stressed, the balance will be broken, the physiological and biochemical functions of the plant cell membrane will be disturbed, and the production of reactive oxygen species will increase (Polle 2001). Concerning

Table 4
List of genes involved in stress response obtained by reference assembly. In the regulation column is reported the Log_2FoldChange mean value of the comparisons considered in each signature.

Gene	Description	Gene Name	Regulation (Log_2FoldChange)
Sensitive Signature			
Bo1g117540	Peptidase A1 domain-containing protein	106,315,440	−3.53
Bo3g001410	RING-type domain-containing protein	106,336,016	1.93
Bo3g132570	Abscisic acid-activated signaling pathway	106,336,161	−2.89
Bo3g134720	TCP domain-containing protein	106,335,966	−1.84
Bo4g014570	Remorin_C domain-containing protein	106,337,585	1.41
Bo8g091960	Remorin_C domain-containing protein	106,307,888	1.45
Bo9g004450	Response to abscisic acid	106,319,095	1.35
Bo5g126420	Dynein light chain	106,343,361	4.95
Bo01141s010	Auxin response factor	106,320,938	−3.52
Bo1g006950	Response to auxin	106,297,419	−5.32
Bo7g095650	Auxin-responsive protein	106,303,757	−1.65
Bo7g119560	Response to auxin	106,304,001	−6.30
Tolerant Signature			
Bo4g190030	Abscisic acid receptor	106,337,293	−7.96
Bo7g075740	Abscisic acid receptor	106,301,784	−1.72
Bo1g016790	Auxin-responsive protein	#N/D	−2.29
Bo2g134160	Auxin efflux carrier component	106,327,550	−1.67
Bo3g086850	Xyloglucan endotransglucosylase/ hydrolase, EC 2.4.1.207	106,336,417	−4.19
Bo8g112780	LOB domain-containing protein	106,310,345	4.01
Intersection Signature			
Bo4g130780	Cytochrome P450 CYP83A1	106,342,466	5.01
Bo6g083440	Enoyl reductase (ER) domain-containing protein	106,298,631	−4.72
Bo2g052680	C2H2-type domain-containing protein	106,320,995	1.45
Bo3g001410	RING-type domain-containing protein	106,336,016	−1.54
Bo3g018500	RING-type domain-containing protein	106,332,045	−2.45
Bo3g132570	FAS1 domain-containing protein	106,336,161	2.33
Bo3g134720	TCP domain-containing protein	106,335,966	1.78
Bo4g038720	EID1-like F-box protein 3	#N/D	−4.97
Bo8g080510	Dehydrin	106,311,856	−7.37
Bo1g103470	Auxin-responsive protein	106,325,843	−2.66
Bo3g179630	Auxin efflux carrier family protein	#N/D	−1.58
Bo5g030850	Auxin efflux carrier family protein	106,293,889	−4.50
Bo7g084150	Protein LAZY 1	106,304,822	−4.89

transcriptomic analysis, we have conducted a comprehensive analysis comparing de novo whole transcriptome sequencing with reference genome analysis employing TO1000 (https://www.ncbi.nlm.nih.gov/datasets/genome/GCA_000695525.1/). Remarkably, the de novo analysis resulted in an absolute highest number of DEGs with respect to the reference method when taking into account all the comparisons analyzed. The highest number of DEGs detected by the de novo approach can be explained considering the different approach of the two methods in gene expression quantification. A large number of paralogous sequence reads from members of the same gene family are often de novo assembled and quantified. Furthermore, the de novo approach allows to estimate the levels of genes that are lowly expressed, have long CDSs, or belong to large gene families, resulting in a higher number of genes that could be differentially expressed when comparing different samples (Lee, Na, and Park 2021). This observation can provide substantial support for our findings regarding the greater number of differentially expressed genes (DEGs) when comparing the de novo method to the reference genome-based approach. In our specific context, although the reference genome employed was one of the most extensively annotated for the *B. oleracea* species, it still offered a limited set of annotations. Notably, we employed as reference genome the TO1000 (BOL), derived from Brassica oleracea var. oleracea. Moreover, as it was highlighted (Fu et al. 2018), de novo assembly often uncovers a significant abundance of transcript isoforms. In order to highlight the transcriptomic response differences to drought stress we intersected the DEGs obtained by the cultivars with the same behavior resulting in three different signatures. The above mentioned DEGs were then enriched for Gene Ontology Biological Processes and KEGG pathways. Firstly, our analysis revealed that within the sensitive reference signature 7 genes play vital roles in abscisic acid (ABA) response and regulation. The phytohormone abscisic acid (ABA) controls various aspects of plant growth throughout development. During vegetative growth, its major role is to mediate adaptive responses to various adverse environmental conditions. Its critical role in the adaptation or acclimation to drought, freezing, and high salinity is well documented (Schroeder, Kwak, and Allen 2001; Xiong, Schumaker, and Zhu 2002). In this work Bo1g117540 resulted downregulated, it encodes a peptidase with aspartic-type endopeptidase activity involved in responding to water deprivation and drought avoidance. Furthermore, the gene Bo3g134720, also downregulated, encodes the TCP14 transcription factor involved in various growth and development processes. Several studies by different authors have consistently associated TCP transcription factors primarily with ABA response and stress applications (Manna et al. 2021; Liu et al. 2022; Huang et al. 2022). Drought stress in plants involves various proteins, with dynein light chain (DLC) playing a crucial role as part of the dynein complex—a motor protein connected to myosin and kinesin. DLC facilitates ATP hydrolysis for mechanical motion, contributing to essential cellular processes (Wilson et al. 2001; Havecker, Gao, and Voytas 2005). Among the upregulated genes in this signature we have found the Bo5g126420 gene which encodes a DLC. This finding aligns with previous research on cotton, rice, Arabidopsis, and tomato, where DLC and microtubule-based process genes were studied in relation to development, abiotic stresses, and phytohormone treatments (Cao, Li, and Lv 2017; Li et al. 2019; Hu et al. 2021; Chun et al. 2021; Wang et al. 2022). Auxin plays an important role during abiotic stress-induced changes in the root and developmental modifications to root system architecture (RSA), is vital for tolerance to water deficiency (Korver, Koevoets, and Testerink 2018) We have found four genes implicated in auxin regulation, all downregulated. The downregulation of Auxin response factors (ARFs) such as Bo01141s010 and Aux/IAA Proteins such as Bo7g095650 show the interruption of auxin uptake and the braking of the plant growth due to the water absence. Consistently to what was found in the sensitive reference signature, genes involved in auxin transport and regulation have been found down-regulated in the de novo sensitive signature. In detail, auxin transporter-like protein 3 involved in proton-driven auxin influx was down-regulated in de novo sensitive signature. It mediates the formation of auxin gradient in plants by contributing to the loading of auxin in vascular tissues and facilitating acropetal (base to tip) auxin transport within inner tissues of the root apex. The down regulation of auxin transporter-like protein 3 suggests a slowdown of auxin transport through the plant with a consequent negative effect on root development and architecture, thus leading to drought stress suffering in sensitive plants. The hypothesis is also supported by the down-regulation of auxin-responsive protein SAUR50 involved in plant growth by promoting cell elongation (Ren and Gray 2015). The abscisic acid (ABA) signaling, and regulation resulted strongly impaired by water deficiency in sensitive plants. In fact an up-regulation of protein phosphatase 2C 78, a negative regulator of ABA signaling for stomatal closure in leaves and a negative regulator of response to drought (Zhang et al. 2012;

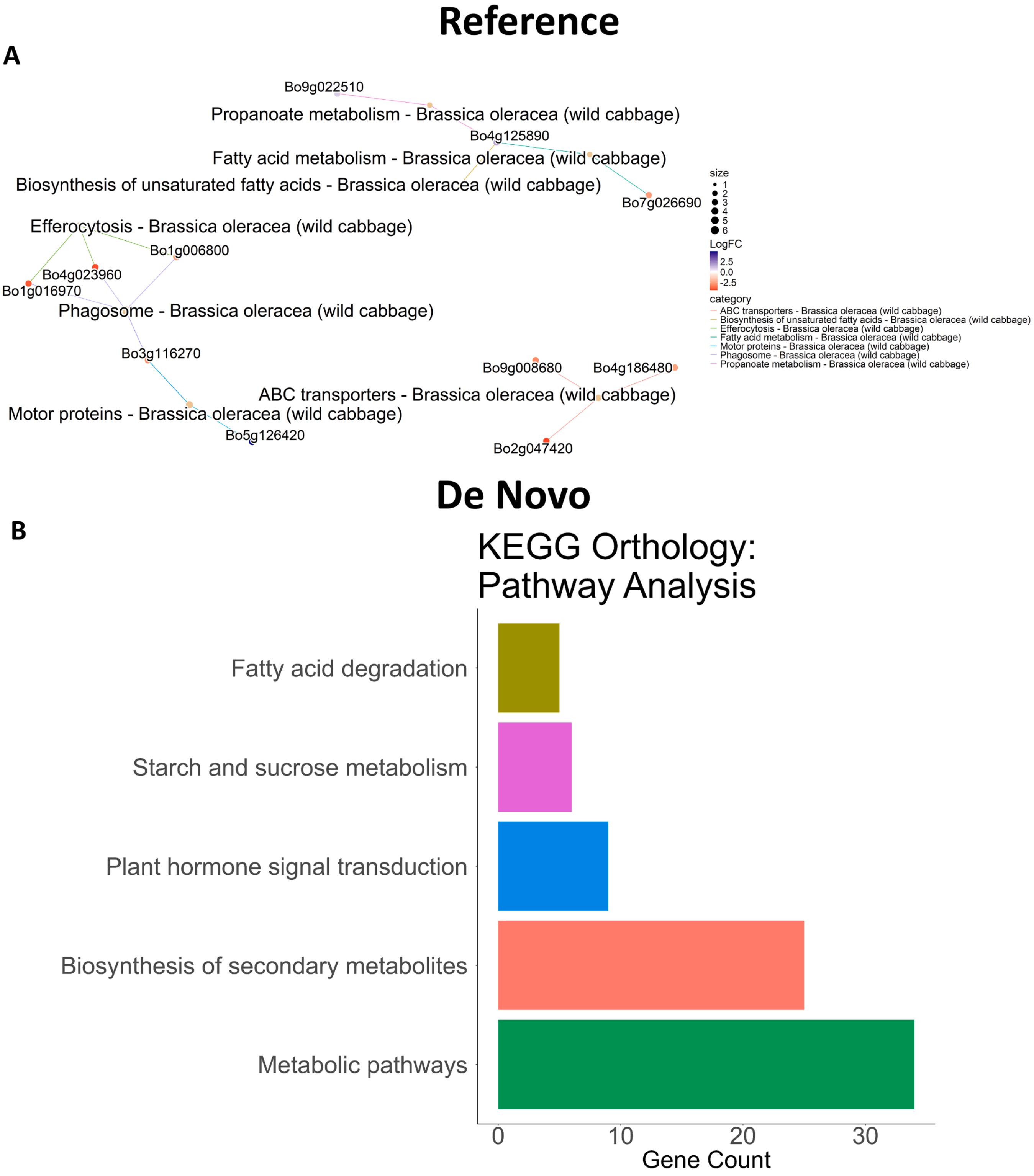

Fig. 7. Pathways network of sensitive signature with reference genome (A) and de novo analysis.

Bhaskara, Nguyen, and Verslues 2012), was measured. Furthermore, abscisic acid receptor PYR1 resulted down-regulated. PYR1 has been reported to promote drought tolerance (Ge et al. 2018). Finally, the production of glucosinolates is also affected by drought stress since a down-regulation of cytochrome P450 83A1-like was measured. It is involved in the biosynthesis of both short-chain and long-chain aliphatic glucosinolates (Hemm, Ruegger, and Chapple 2003). Secondly, in tolerant reference signature we have found two downregulated genes correlated to ABA signaling pathways with respect to control samples. In particular Bo4g190030 and Bo7g075740, identified with Uniprot as orthologues of respectively PYL6 and PYL1, are ABA receptors. Both of them are required for ABA-mediated responses such as stomatal closure and germination inhibition. Other three downregulated genes, instead, are involved in auxin regulation. In particular, Bo1g016790 is an Aux/IAA protein that functions as repressors of early auxin response genes at low auxin concentrations, its downregulation makes us think

Table 5
List of genes involved in stress response obtained by de novo assembly. In the regulation column is reported the Log$_2$FoldChange mean value of the comparisons considered in each signature.

Cluster ID	Descritpion	Swissprot ID	Regulation (Log$_2$FoldChange)
Sensitive signature			
8815.8016	probable protein phosphatase 2C 78	Q9FIF5	6.92
8815.41697	Indole-3-acetic acid-amido synthetase GH3.6	Q9LSQ4	4.38
8815.44239	Cytochrome P450	P48421	−3.80
8815.26346	Abscisic acid receptor PYR1	O49686	−3.88
8815.2881	Auxin transporter-like protein 3	Q9CA25	−5.61
8815.52826	Auxin-responsive protein SAUR50	O65695	−5.20
Tolerant signature			
8815.28341	ABSCISIC ACID-INSENSITIVE 5-like protein 6	Q9M7Q3	2.08
8815.26738	Protein phosphatase 2C	P49598	1.88
8815.28482	Magnesium dechelatase SGRL, chloroplastic	Q94AQ9	–1.10
8815.32878	Transketolase-1, chloroplastic	Q8RWV0	–1.21
8815.44828	Auxin-responsive protein IAA28	Q9XFM0	−1.57
Intersection signature			
8815.31925	Cytochrome P450 83A1	P48421	3.65
8815.51356	9-cis-epoxycarotenoid dioxygenase NCED3, chloroplastic	Q9LRR7	−2.96

about a higher auxin intake of the plant. Bo2g134160 is an orthologues of PIN4, PIN3 and PIN7 of Arabidopsis thaliana. All of them are classified as auxin efflux carriers, this suggests that its downregulation can be involved in higher concentration of auxin in the tolerant plants. Furthermore, Bo3g086850 Catalyzes xyloglucan endohydrolysis (XEH) and/or endotransglycosylation (XET) (Gonzalez-Guzman et al. 2012), participating in cell wall construction. Moreover, we have found the gene Bo8g112780 upregulated, this gene encodes Lateral Organ Boundaries (LOB) domain-containing protein and it has two orthologues in Arabidopsis thaliana (Ye et al. 2021), LBD1 and LBD11. As seen by Ye et al. in 2021, LBD genes overexpression promotes cell growth and cell division enhancing secondary growth suggesting a resistance to drought stress. As regards the de novo tolerant signature, a gene encoding ABSCISIC ACID-INSENSITIVE 5-like protein 6 (ABF3) resulted up-regulated. It binds to the ABA-responsive element (ABRE) mediating stress-responsive ABA signaling. In detail overexpression of ABF3 or ABF4 in *Arabidopsis* resulted in ABA hypersensitivity and enhanced drought tolerance with changes in the expression levels of a number of ABA- or stress-regulated genes (Kang et al. 2002). Furthermore, a gene encoding for protein phosphatase 2C 37-like resulted also up-regulated. This protein prevents stomata closure by inactivating the S-type anion efflux channel SLAC1 and its activator SRK2E (Lee et al. 2009). Interestingly, homologous of both ABSCISIC ACID-INSENSITIVE 5-like protein 6 and protein phosphatase 2C 37-like were found up-regulated in *Arundo donax* when subjected to high levels of salt stress (Sicilia et al., 2019), confirming the importance of ABA signaling in the tolerance of plants to stresses leading to osmotic imbalance. Finally, a gene encoding magnesium dechelatase SGRL was found among the down-regulated genes in the tolerant signature. It is involved in chlorophyll degradation in the chlorophyll-protein complexes of photosystem I (PSI) and photosystem II (PSII) (Shimoda, Ito, and Tanaka 2016). Contributes to abiotic stress-induced chlorophyll degradation and leaf yellowing during vegetative plant growth(Sakuraba et al. 2014). This result is consistent with the high level of SPAD index measured in tolerant plants when subjected to water deficit conditions suggesting that stress tolerance is related with regular photosynthetic processes.

Through the intersection of differentially expressed genes between tolerant and sensitive conditions we identified the intersection signature. In this signature we have found 7 genes involved in ABA regulation (4 down and 3 up-regulated) and 4 in auxin regulation (all down-regulated). In particular, the gene Bo2g052680 (Yihua Liu, Khan, and Gan 2022) encodes for a C2H2-type domain-containing protein; these proteins have been shown to respond to abiotic stress in plants. It is involved in negative abscisic acid regulation as its orthologues in Arabidopsis thaliana (ZFP4) (Joseph et al. 2014). The gene Bo3g13472, already seen downregulated in sensitive signatures, in intersection signature results upregulated. Its orthologue in Arabidopsis, called TCP14 (Zhang et al. 2019), regulates germination. Bo5g030850 and Bo3g179630 are two auxin efflux carriers which result in being downregulated in tolerant versus sensitive plants, suggesting that drought stress tolerant plants retain more auxin than sensitive ones.

In our study, we observed significantly higher expression of the Bo4g130780 gene involved in GO such as glucosinolate biosynthetic process [GO:0019761]; response to insects [GO:0009625]. This gene, identified as cytochrome P450 83A1 (Guo et al. 2019) monooxygenase (CYP83A1), plays a pivotal role in glucosinolate biosynthesis suggesting a modulation of glucosinolate production, particularly in the biosynthesis of aliphatic glucosinolates, in response to drought stress. The cytochrome P450 superfamily (Xu, Wang, and Guo 2015) in fact brings to the synthesis of numerous secondary metabolites that function as growth and development signals to plants. Furthermore, our findings are corroborated by different studies (Yi et al. 2015; Salehin et al. 2019), where they observed reduced drought stress resistance in Arabidopsis as a consequence of the loss of function of the CYP83A1 gene. The importance of cytochrome P450 83A1 is highlighted by the fact that it was found strongly up-regulated also in the de novo intersection signature. Another identified gene, Bo6g083440, orthologues of ADH1 of Arabidopsis thaliana, encodes an alcohol dehydrogenase enzyme. We observed a significantly lower expression of this gene in tolerant plants. This finding aligns with a previous research (Li et al. 2021), who detected the upregulation of genes in B. napus sensitive to drought stress, resulting in increased fatty acid degradation and a reduction in oil content.

Among the examined genes, Bo9g041010 (a homolog of Arabidopsis thaliana Transcription factor bHLH112, SwissProt Q94JL3) emerged as a common denominator in both tolerant and sensitive cultivars, exhibiting a discernible trend in expression. Specifically, it displayed upregulation in tolerant cultivars and downregulation in sensitive ones. AtbHLH112, a nuclear-localized protein, is known to undergo induction of nuclear localization in response to salt, drought, and abscisic acid (ABA). Gain- and loss-of-function analyses have highlighted a positive correlation between the transcript level of AtbHLH112 and salt and drought tolerance. Additionally, AtbHLH112 facilitates the upregulation of POD and SOD genes, enhancing the scavenging ability of reactive oxygen species (ROS) (Liu et al. 2015).

5. Conclusion

Transcriptomic analysis stands as a cornerstone in unraveling plant stress responses, offering a pivotal avenue for advancing stress-tolerant crop development—an imperative for sustainable agriculture amidst global environmental shifts. The vast genetic diversity inherent in the *B. oleracea* complex species ($n = 9$) provided a rich resource for identifying genotypes pivotal in elucidating genes and pathways crucial for drought tolerance in *B. oleracea* crops. By employing two transcriptomic methodologies—reference and *de novo*—we delved into the intricate realm of drought stress in *B. oleracea* crops, garnering nuanced insights and broadening our understanding of genes pivotal to water stress. In particular, the scarcity of robust annotations for *B. oleracea* in the reference analysis underscored the challenge posed by limited functional annotations, profoundly impacting our analysis. Conversely, the de novo approach unveiled an extensive gene repertoire, shedding light

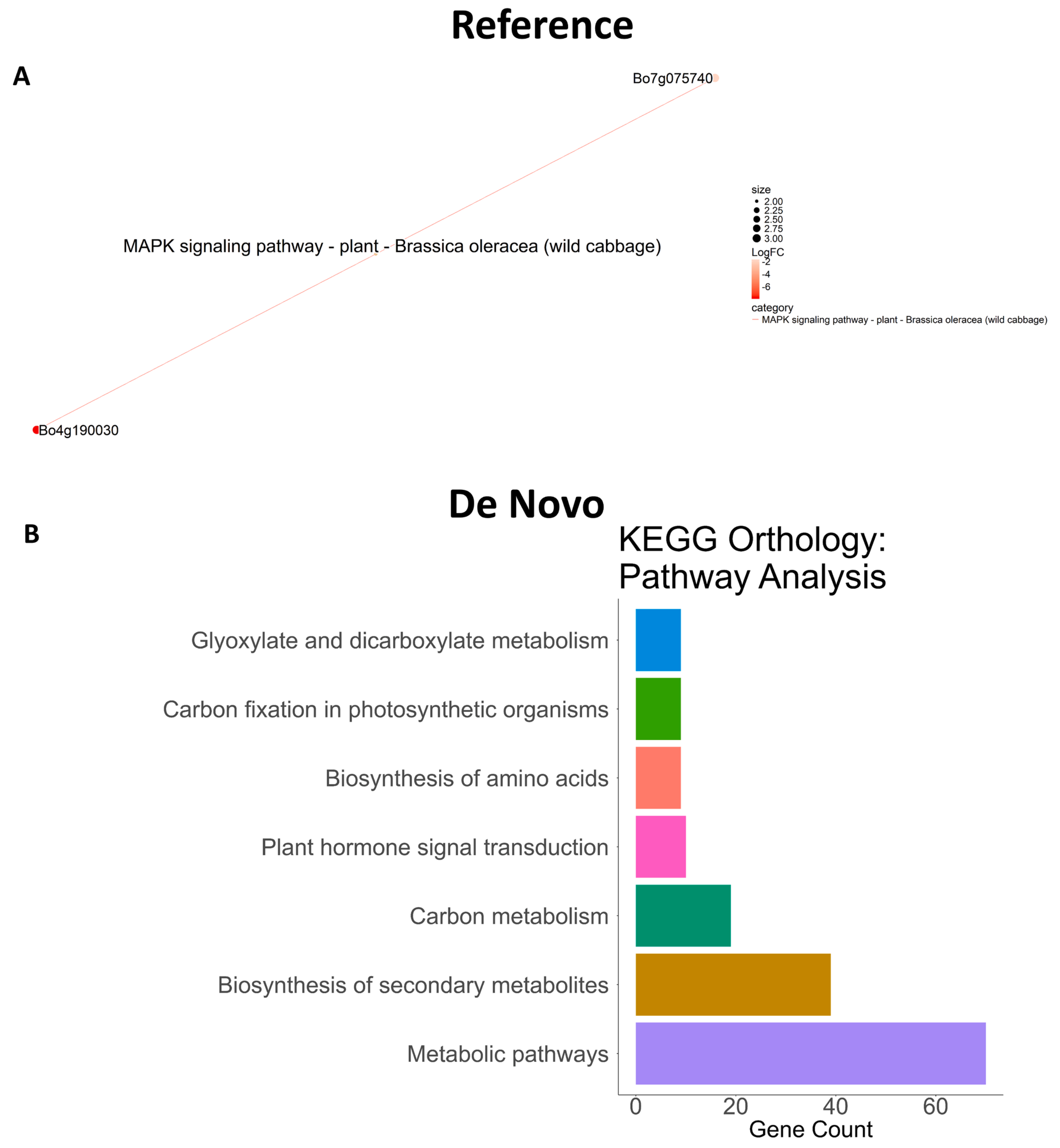

Fig. 8. Pathways network of tolerant signature with reference genome (A) and de novo analysis (B).

on crucial metabolic pathways pivotal in stress adaptation, particularly in plant hormone signal transduction mechanisms. Here, the roles of auxin and abscisic acid emerged as central in stress perception and adaptation. A noteworthy discovery surfaced in the identification of a potential candidate gene for water stress tolerance—transcription factor bHLH112—significantly associated with drought tolerance. The adoption of a dual reference/de novo transcriptome assembly approach yielded a more comprehensive dataset compared to a singular approach, enabling an unbiased interpretation of outcomes. Lastly, leveraging the *ex situ* conserved genetic diversity by the Di3A of the University of Catania in investigating drought stress responses across various *B. oleracea* complex species ($n = 9$) proved instrumental. This approach not only facilitated the exploration of latent traits but also unearthed forgotten alleles, shedding light on the domestication processes of B. oleracea crops and guiding future endeavors in crop improvement.

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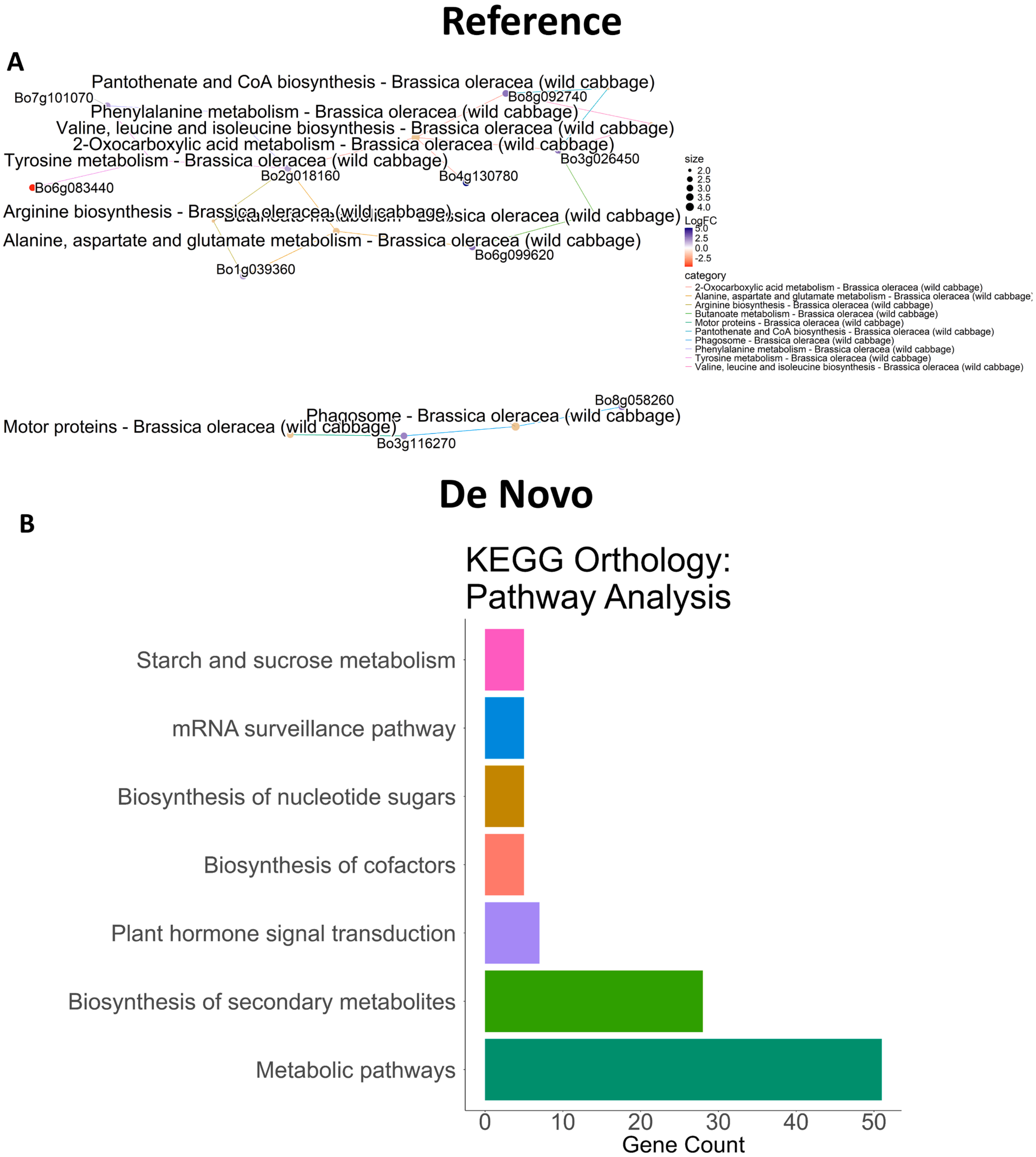

Fig. 9. Pathways network of intersection signature with reference genome (A) and de novo analysis (B).

CRediT authorship contribution statement

Grete Francesca Privitera: Writing – review & editing, Writing – original draft, Software, Resources, Methodology, Formal analysis, Data curation. **Simone Treccarichi:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Roberta Nicotra:** Writing – original draft, Software, Formal analysis. **Ferdinando Branca:** Supervision, Methodology, Funding acquisition, Conceptualization. **Alfredo Pulvirenti:** Writing – review & editing, Supervision, Methodology, Data curation. **Angela Roberta Lo Piero:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Angelo Sicilia:** Writing – review & editing, Writing – original draft, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.stress.2024.100657.

Data availability

The Bresov drought stress transcriptome was submitted to NCBI Sequence Read Archive under the accession number PRJNA973227.

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