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Comparative expression analysis of stress-inducible candidate genes in response to cold and drought in tea plant (Camellia sinensis (L.) Kuntze)

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Cold and drought are major factors reducing the yield and geographical distribution of most horticultural crops worldwide. Both cold and drought are lead to decreasing of water potential of plant tissues and induce ROS accumulation causing severe damage to various cellular components such as altering membrane lipid composition due to excess accumulation of malondialdehyde (MDA), structural proteins and enzymes. Plant responses to the stresses are complex and complicated especially in perennial woody crops and hundreds of genes are involved. To develop cultivars tolerant to both cold and drought it is necessary to find genes involved in both stress-tolerance to develop genetic markers.

Tea plant (Camellia sinensis L.) is one of the most important economic crops in China, India, Sri Lanka, Kenya, as well as Caucasian countries (Turkey, Georgia, Russia and Azerbaijan). This perennial woody evergreen crop grows in 60 countries on 5 continents, from 49° N in Ukraine to 33° S in South Africa (Turkozu and Sanlier 2017). Tea plantations in most countries are affected by drought and cold stress that reducing the yield significantly and decreasing the distribution of the crop to colder areas.

Previous studies on cold and drought tolerance have identified several regulons comprising of transcription factor (TF). Many transcription factors and metabolite genes were showed to be involved in both cold and drought responses of plants. For example functional characterization of the key cold regulators ICE, CBFs as well as DHNs transcription factors are participated in both cold and drought as well as in the other abiotic stresses (Ding et al. 2015; Yin et al. 2016; Ban et al. 2017; Hu et al., 2020). Many genes involved in ABA-independent responsive pathway and the bZIP-mediated ABA-dependent pathway (Wang et al., 2012; Ban et al., 2017) participated in the tolerance to cold and to drought. The overexpression of CsbZIP6 in Arabidopsis resulted in hypersensitivity to several abiotic stresses (Cao et al., 2015).

Various bHLH factors could enhance plant tolerance to chilling stress by activating stress-responsive genes and ROS cleavage pathway (Sun X. et al 2018). G-type lectin S-receptor-like serine/threonine protein kinases are referred as positive regulators of plant salt resistance (Sun X. L. et al., 2013), and therefore may have impact on cold tolerance. It was reported that SnRK1 is highly conservative sensor which regulates gene expression in response to stress and energy depletion (Cho Y. H. et al. 2012). Hexokinases are sugar sensors which help to maintain homeostasis during various treatments (Sarowar S. et al. 2008). Overexpression of ERF factors leads to increasing cold tolerance (Sun X. et al. 2019). WRKY family has a potential role of being cold-inducible factors. Dehydrin ShDHN, besides its upregulation during cold treatment, also detected to improve resistance to other abiotic stresses in tomato (Liu H. et al. 2015). It was revealed that ZmLEA3 is involved in protection from low temperature stress (Liu Y. et al., 2011).

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Other proteins from multiple classes also having various impacts during abiotic stresses in dicotyledonous plants. For instance, well-known factor ICE1, heat-shock protein HSP70, antioxidant genes (GST, POD) which regulate ROS metabolism during stress treatment. However, we still lack of complex picture related to interactions between core network and their downstream-regulated target proteins. Thus, we need to keep searching new evolutionarily conservative and species-specific genes related to tress response. Hence, it becomes essential to assess these genes as the possible markers to identify naturally occurring cold and drought tolerant tea genotypes with diverse mechanisms from the tea germplasm for breeding new tolerant cultivars.

In this study we combine literature data with our interspecific transcriptomic analysis (Arabidopsis thaliana and Solanum lycopersicum) for choosing relevant genes related to cold tolerance. We phenotypically screened a panel of Caucasian tea genotypes for cold and drought tolerance. Further expression analysis of 47 genes (earlier reported possible stress-markers and new in silico identified candidate genes) was performed in the most tolerant genotype to reveal the involvement of these genes in cold and drought responses and to find correlations between both responses. We have analyzed the expression of the candidate genes under long-term stress induction and following recovery stages in cold and drought to reveal their involvement and to find overlapped Cold/Drought responses. For studied genes we built gene network of the corresponding homologues for Arabidopsis thaliana which reflects our experimental and computational results.

Materials and methods

In silico search of candidate-genes, network reconstruction and layout

For revealing candidate genes which significantly increase their expression levels we performed interspecific analysis of experiments with transcriptomic data of cold treated plants stored in GEO NCBI database (ncbi.nlm.nih.gov/geo/ , Barrett T. et al. 2012). We used data GSE103964, GSE112225, GSE116964 for Arabidopsis thaliana and GSE78154 for Solanum lycopersicum. We calculated fold changes of gene expression during cold treatment and assigned ranks of genes according to their quartile of upregulation (from 1 to 4). Next, we compared top quartile genes between Arabidopsis thaliana and Solanum lycopersicum using standalone BLAST (Camacho C. et al. 2009). As a result, we revealed 9 orthologs groups of genes which detected as genes with highest rank in both species. We found corresponding homologues of 9 candidate genes for Camellia sinensis using BLAST against Tea Plant Information Archive database (Xia E. H. et al. 2019, ID started with «TEA» in Table 2). We founded corresponding Arabidopsis thaliana orthologs of Camellia sinensis sequences from Li et al. (2019) using best-scored BLAST result. For genes selected from literature we evaluated Arabidopsis thaliana orthologs presented in TAIR database (Lamesch P. et al. 2012). Primers were designed using publicly available service PrimerQuest (eu.idtdna.com/Primerquest) with default parameters and amplicon size between 100 and 250 n.p. After that, we revised quality of obtained primers using service Multiple Primer Analyzer by Thermofisher Scientific. For our gene set we reconstructed network using String database (https://string-db.org; Szklarczyk D. et al. 2019) with following attributes: Textmining/Experiments/Databases interactions and threshold of interaction score = 0.15. For further layout and visualization we used Cytoscape application (cytoscape.org; Shannon P. et al. 2003).

Our analysis involved set of 52 genes, which include 9 de novo predicted genes from transcriptomic data analysis and 43 genes from recent articles related to cold tolerance of *Camellia sinensis*. Our analysis of available literature led to the inclusion following genes: bHLH factors (10), GsSRK (2), SnRK1 (3), hexokinases (3), ERF (3), WRKY (2), dehydrins (2), late embryogenesis abundant proteins (2), and others (CBF1, ICE1, ZAT, HSP70, PRP, CIP, PEI, TLP, POD, GST, BMY, ALE2, FLS2). Also, using interspecies transcriptome analysis we stressed out 9 orthologs which were highly upregulated in both species during

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cold treatment: 2 galactinol synthases (GOLS1 and GOLS3); glycine-rich RNA-binding protein 3 (GR-RBP3); Xyloglucan endotransglucosylase/hydrolase protein 22 (XTH22), zinc finger protein RHL41, histone methylase SKB1, pectinesterase inhibitor PME41, dehydration response element-binding protein DREB26, Protein kinase superfamily protein ARCK1.

Plant material

The experiments on cold and drought induction were carried out using three-year-old plants Quimen population. Plants were grown in 2 liter pots filled with brown forest acidic soil (pH = 5.0). For each assessed parameter, 2nd, 3rd and 4th mature leaves were used for samplings. Experimental treatments with these plants were conducted during two years 2019-2020.

Stress induction and phenotypical screening for tolerance

Cold stress was induced in cold chambers HF-506 (Liebherr, Denmark) as follows: decreasing the temperature by 0- +2 °C for 10 days (cold treatment) to reveal the mechanisms of cold acclimation. After that temperature was gradually increased till +10 ^{o}C during 10 days (Recovery-Cold treatment).

Drought stress was induced in laboratory climatic chamber by gradually decreasing the watering till 15-17 % of water content in soil (comparing with control 28-30%) during 10 days (drought treatment) to reveal the mechanisms of drought acclimation. After that, watering was gradually increased till 28-30 % during 10 days (Recovery-Drought treatment).

Gene expression analysis

Total RNA was extracted from the third mature leaf in three biological replicates by the guanidine method with sorption on silica columns, according to the manufacturer's protocol (Biolabmix, Novosibirsk, Russia). Actin was taken as a reference gene and results were quantified using a Light Cycler 96 analyzer (Roche). The relative gene expression level was calculated by the K.J. Livak and T.D. Schmittgen (2001) using following algorithm: 2-ΔΔCq, where:

$$\Delta\Delta Cq = (Cq_{gene\ of\ interest} - Cq_{internal\ control})_{treatment} - (Cq_{gene\ of\ interest} - Cq_{internal\ control})_{control}$$

Statistical analysis

All analyses were repeated three times during two years with three to five biological replicates. Statistical analyses were carried out using XLSTAT software. Student t-test, principal component analysis and Pearson's correlation tests and Wards-clusterization were performed to evaluate data and confirm the significant differences (at the level $P<0.05$) between the genes expression profiles and respective treatments.

Results and Discussion

Generally, Cold response was more active in our study then drought response. More genes with the highest expression levels were induced in Cold comparing to Drought. The genes significantly upregulated under both Drought and Cold were: HSP70, DHN1, bHLH116, BMY, bHLH102, GR-RBP3, ICE1, GOLS1, GOLS3 indicating their possible roles in both stress responses. The genes, differentially upregulated in Cold were SUS1, GST, SnRK1.2, HXK1, HXK2, bHLH43, bHLH79, bHLH7, bHLH93. The genes differentially upregulated under Drought were RHL41, CAU1, Hydrolase22. The transcripts of CIP were mostly accumulated in RecCold and RecDrought; the transcripts of PME41 were mostly accumulated in RecDrought indicating the possible important role of these two candidate genes in plant recovery after stress. Taken together, these genes may be excellent resources for tea defence against environmental stresses. These results provide valuable information and robust candidate genes for future functional analysis aimed at improving the drought and cold tolerance of the tea plant.

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