Tea Tree NOX Amplification of gene families and their expression analysis in tea plant stress resistance

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Abstract Reactive oxygen species (ROS), as key signaling molecules in response to stress, play an important role in plant signal transduction and developmental regulation. Plasma membrane NADPH oxidase (NOX) is the main source of ROS production and plays a crucial role in plant-pathogen interactions. This study systematically analyzed *the NOX* gene family in the tea plant genome, identifying nine *NOX* (*CsNOX*) genes. Phylogenetic, gene structure, conserved domain, motif, and collinearity analyses revealed that the tea plant *NOX* gene family can be divided into three subfamilies, exhibiting high sequence conservation and containing three pairs of repetitive gene segments. The ratio of nonsynonymous mutation rate to synonymous mutation rate (Ka/Ks) is less than 1. Promoter element analysis showed that tea plant *NOX* genes contain not only numerous stress-response elements but also various hormone-related response elements. Expression pattern analysis revealed that tea plant *NOX* genes exhibit tissue-specific expression characteristics and participate in various abiotic stress response processes. This study, through bioinformatics analysis of the tea plant *NOX gene family, provides a theoretical basis and reference value for further exploration of its functions.*

Keywords tea plant; NOX; ROS; evolution

Foreword

NADPH oxidase (NOX) is a nicotinamide adenine dinucleotide phosphate-dependent oxidase that can use electrons provided by NADPH to catalyze the generation of superoxide anions from oxygen molecules. It is an important enzyme in organisms that produces reactive oxygen species (ROS) and is widely distributed in nature. This enzyme was first discovered in phagocytes of the human immune system and exists in mammalian neutrophils and plant cells. It is a multi-enzyme complex located on the cell membrane. NOX is mainly composed of two parts: the core enzyme part (including p40phox, p47phox, p67phox, p22phox and gp91phox) and small molecular weight guanylate-binding proteins (such as Rac2 and Rap1A) [1]. In the resting state, NOX remains inactive. When cells are stimulated by external factors, the complex composed of the core enzymes p40phox, p47phox and p67phox interacts with Rac -GTP through the proline-rich tail of p22phox, which leads to a conformational change of gp91phox, thereby activating NOX and catalyzing the generation of reactive oxygen species from oxygen molecules [2].

Reactive oxygen species (ROS) can act as signaling molecules and play an important role in the stress response of plants. Studies have shown that when plants are subjected to stress, the burst of ROS is a common phenomenon [3], and the ROS produced by plants in response to stress is mainly generated by NOX catalysis [4-6]. When plants are subjected to external signal stimulation, NOX rapidly regulates the level of ROS in the plant through changes in its own conformation in response to biotic or abiotic stress. Under biotic stress, pathogen invasion will promote the enhancement of NOX activity, leading to an increase in the concentration of ROS in the plant. Subsequently, ROS directly kills pathogens through its oxidative toxicity or induces programmed cell death, thereby inhibiting the further spread of pathogens [7]. Under abiotic stress, Arabidopsis *thaliana AtNOX C and* AtNOX *D participate* in the mechanical damage response by regulating the level of ROS [8,9]. Under salt stress, NOX activity in maize leaves decreases, leading *to* a reduction in the level of ROS in cells, which in turn affects the elongation growth of leaves [9].

In plants NOX also has other biological functions, for example, in Arabidopsis thaliana, NOX participates in ABA signal transduction [10]; in rice, the NOX gene shows obvious spatiotemporal and climatic regulation characteristics in response to drought (*OsNOX1-3, OsNOX5, OsNOX9, OsFRO1 and OsNOX6*) , salt *stress* (*OsNOX2* , *OsNOX8* , OsFRO1 , OsFRO7 , OsNOX1 , OsNOX3 , OsNOX5 *and* OsNOX6 *)* and *high* temperature *treatment* (OsNOX5-9 , OsNOX1-3 and *OsFRO1)* [11]. In addition , *NOX* Besides participating in responses to abiotic stress, it also participates in the regulation of plant growth and development. For example, in maize leaves... NOX It participates in the elongation process of maize

leaves[12]; Arabidopsis thaliana *AtNOXC* It participates in cell elongation and root hair growth processes [13-15]; AtNOXH and AtNOXJ It is essential for pollen tube growth and fertilization in Arabidopsis thaliana [16-19]. Further research has shown... NOX It also participates in a series of developmental processes, including seed germination, growth and stomatal closure [20].

Tea (*Camellia sinensis*) is one of the important economic crops in China, Japan, India and Africa. It is suitable for growing in warm and humid environments. However, adverse factors such as high temperature, drought, high salt and pests often have an adverse effect on the growth and development of tea trees, thereby reducing the yield and quality of tea. In recent years, with the continuous progress of sequencing technology, the genome sequencing of many tea tree varieties has been completed one after another. However, as of now, there are no reports on the study of *NOX genes in tea trees* [37]. Therefore, we identified *NOX genes* in the tea tree genome and conducted a comprehensive analysis of their phylogenetic relationship, gene structure, protein structure, chromosome distribution, homology, promoter elements and expression characteristics. Through these studies, we have preliminarily revealed the functional mechanism of *NOX* genes in tea trees to resist adverse stress, providing a theoretical basis and reference for subsequent functional verification and resistance breeding research.

Materials and Methods

1: Tea Tree NOX Identification of genetic family members

First, the domains of Arabidopsis *NOX* gene family members were predicted and analyzed using SMART (Simple Modular Structure Study Tool, https://smart.embl.de/). The results showed that the domain unique to *NOX genes was* NADPH_Ox (PF08414) [21] . Then, the hidden Markov file of the domain NADPH_Ox was downloaded from the Pfam database (http://pfam.Xfam.org) [22] , and the genome and proteome data of tea plants were obtained from the TPIA (tea plant information archive) database. Then, the hidden Markov model (HMMsearch , E value set to 10-10) and the basic local alignment search algorithm (BLASTP, E value set to 10-10) were used to search for NADPH gene family members in the tea plant proteome. The obtained protein sequences were then used to predict and verify the domains using the SMART tool to ensure that all selected tea plant *NOX* genes contained the NADPH_Ox domain. The identified *NOX* gene in the tea plant was used for further analysis.

2: Tea Tree NOX Phylogenetic analysis of gene families

First, the obtained NOX protein sequence was aligned using Muscle 5 in TBtools . Then, the sequence was pruned using the Multiple Alignment TrimAL module. Finally, a phylogenetic tree was constructed using the IQ-Tree wrapper module, and the results were visualized using iTOL (https://itol.embl.de/) [23] .

3: Tea Tree NOX Statistical analysis of physicochemical properties, gene structure analysis and motif analysis

The physicochemical properties of the *NOX gene* in tea were statistically analyzed using the Protein Parameter Calc module in TBtools ; the motif of the NOX protein sequence in tea was searched using the Simple MEME Wrapper module [23,24] . Gene annotation files of tea were downloaded from the TPIA database and visualized using TBtools ' Visualize function. gene Visualize the *NOX gene structure of tea trees using* the Structure (from GTF/GFF File) module .

4: Tea Tree NOX Analysis of cistropic elements in gene promoter regions

Based on the genomic data and gene annotation files of tea plants, the upstream 2000 bp sequence of the *NOX gene in tea plants was extracted using the* Gtf / Gff Sequence Extract module of TBtools and defined as the promoter region of the *NOX gene. Then, the online tool* PlantCARE

(http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used to perform cis-regulatory element prediction analysis on this promoter region.

5: Chromosomal localization and collinearity analysis of *NOX genes in tea plants*

Based on the gene annotation file of tea plant, the location of the *NOX gene on the chromosome* of tea plant was visualized using the Gene Location Visualize from GTF/GFF module of TBtools . At the same time, using genomic data and gene annotation file, the collinearity relationship between the *NOX gene* of tea plant itself and between tea plant and Arabidopsis thaliana was analyzed using the One Step MCScanX - Super Fast module of TBtools , and the results were visualized using the Multiple Synteny Plot module. In addition, the Ks/Ka value of duplicated genes was calculated using the Simple Ka/Ks Calculate (NG) module [23,25] .

6: Expression analysis of *NOX gene in tea plants*

Gene expression data of the *NOX* gene in tea plants under eight tissues (terminal bud, flower, fruit, young leaf, mature leaf, senescent leaf, root, and stem) and four abiotic stresses (cold, NaCl, drought (PEG) , and methyl jasmonate (MeJA)) were obtained from the TPIA database. These data were then analyzed using TBtools . HeatMap Plate drawing expresses heat map [23] .

Result

1: Identification of NOX genes in tea plants

Through HMMsearch And BLASTP search and SMART Predictive testing identified nine NOX gene family members in the tea plant proteome. These genes encode between 757 and 969 amino acids, with CsNOX 4 encoding the most and CsNOX 9 the fewest . The predicted relative molecular weights range from 86.8 kDa to 110.11 kDa , with CsNOX 4 having the highest molecular weight and CsNOX 9 the lowest . All CsNOX... The predicted isoelectric points of all proteins were greater than 8, and all were basic proteins (Table 1).

2 : Evolutionary analysis of the NOX gene family in tea plants

To further investigate the evolutionary relationships and clustering of the NOX gene family in tea,

aphylogenetic tree was constructed using the protein sequences encoded by NOX genes from tea, Arabidopsis thaliana, rice, soybean, alfalfa , and bird's eye (Figure 1). Based on the topological structure of the phylogenetic tree, the gene families were divided into three subfamilies (Group-I, Group- II, and Group - III) . *The CsNOX 6/9 gene* in tea ... Belongs to Group - I Subfamily, *CsNOX 2 - 5* Belongs to Group - II Subfamily, *CsNOX 1 / 7/8* Belongs to Group - III Sub-family. In Group - II In the subfamily, *CsNOX 2* and *AtNOXE* and *CsNOX 3-5* and *AtNOXI /F* They have high homology; in Group III CsNOX *1 / 7/8* and *AtNOXA /C/D/G* They exhibit high homology. However, in Group I ... In China, *AtNOX 6/9* and *AtNOXJ /H* Although they belong to the same subfamily, they are located on two different branches.

3: Chromosomal localization and collinearity analysis of the NOX gene family in tea plants

Chromosomal mapping results of NOX gene family members in tea plants showed that 9 *CsNOX genes* ... Genes can be located on four chromosomes and two contigs. On *the* fragment (Figure 2). CsNOX *1/2* is located on chromosome 2, *CsNOX 3/4* Located on chromosomes 1 and 2 , *CsNOX 5* Located on chromosomes 1 and 4 , *CsNOX 6/7* Located on chromosomes *1 and* 5 *, and CsNOX 8/9* They were located on Contig 464 and 581 segments, respectively . *CsNOX cells* were distributed over a wide area on the chromosome, and no clustering occurred.

To further investigate *CsNOX* Homologous relationships between genes were analyzed using MCScanX. Collinearity analysis was performed (Figure) 2). The results showed that *the CsNOX content in tea plants...* There are 3 pairs of duplicate gene segments (*CsNOX 1 -CsNOX 7* , *CsNOX 3- CsNOX 5)* . And *CsNOX 6- CsNOX 9). Ka* /Ks analysis of the three pairs of duplicate genes showed that the Ka/ Ks values were all less than 1, and the Ka /Ks values of two pairs of genes were only 0.15 and 0.13 (Table 2), indicating that they were subjected to strong purifying selection during evolution. In the collinearity analysis of Arabidopsis and tea, *CsNOX 4- AtNOXI /F* , *CsNOX 5- AtNOXI /F* and *CsNOX 7- AtNOXC* They have a common origin.

4: Tea tree NOX Gene structure and protein sequence analysis of gene families

Using SMART Predicting CsNOX structure domain and through TBtools Visualization was performed (Figure 3A). A total of five protein domains were predicted: NADPH_Ox (PF08414), Ferric_reduct (PF01794), FAD_binding_8 (PF08022), NAD_binding_6 (PF08030) , and EF - hand . The four domains NADPH_Ox , Ferric_reduct , FAD_binding_8 , and NAD_binding_6 are all CsNOX domains. Common to all proteins. Except for CsNOX 6/9 , the rest of CsNOX... Both have two EF - hand structural domains for

binding with Ca^{2+} · However, in CsNOX 6/9 , NADPH_Ox Two low-confidence EFs were found at the C-terminus of the structural domain - hand Domains (not shown in the figure). Sequences containing four common domains were extracted and multiple sequence alignment revealed CsNOX. The sequences of the protein's four domains are highly conserved (Figure 3C).

Tea Tree NOX The gene structure of the gene family shows that most *CsNOXs* have 12-14 exons, with 14 exons being the most common type, and there are a total of 6 *CsNOXs.* With 14 exons, the most *CsNOX 1 It* has 17 exons, with the fewest being *CsNOX 7/8* . There are 1 or 2 exons. Classified by group, only members of Group - II have a different number of exons (Figure 3B).

To further investigate CsNOX The protein sequence was obtained using TBtools . Motif analysis was performed (Figure 4). A total of 8 different motifs were retrieved in CsNOX , and each motif... Both are CsNOx Shared by all. The Motif logo . The image shows each motif They all have some completely conserved amino acid sites.

5 : Tea trees NOX Analysis of cistropic elements in promoter regions of gene families

CsNOX from tea trees The upstream 2kbp sequence of the gene was used as the promoter region for prediction of cisfunctional elements, and the results are shown in Figure 5. *CsNOX* in tea plants. Genes contain multiple response elements with complex functions, participating in various physiological processes in tea plants, including responses to biotic and abiotic stresses, plant growth and development, and hormone regulation. The most numerous element is... Box 4 , this element is related to the light response. The second most common element is ARE, which is related to anaerobic induction in plants.

6 : Tea Tree NOX Gene family expression analysis under different treatments and tissue-specific expression analysis

From TPIA Downloaded tea tree *CsNOX* Expression data were used to create an expression heatmap (Figure 6). Under drought conditions, *CsNOX 1/4/5/9/2* In the early stages, the expression levels were all upregulated, but gradually decreased with increasing treatment time. Under methyl jasmonate treatment, *CsNOX 1/6* Expression levels gradually increased with increasing processing time, *CsNOX 2 / 7 CsNOX expression levels are high in the early stages and low in* the later stages. Under salt treatment, *CsNOX 3/4/9* The expression level gradually increased over time, *CsNOX 2* Expression levels gradually decreased over *time* . Under cold conditions *, CsNOX 2/3/5/6/8* The

expressiongradually decreased over time.

Tissue expression analysis showed (Figure 7) that *C sNOX 1* This was expressed in all eight organizations, *CsNOX 6/9 Expression* levels were low in all eight tissues. Furthermore *, CsNOX 7/8 CsNOX 3/4* was expressed at relatively high levels in flowers, roots, and stems. The expression levels *of CsNOX* are relatively high in flowers and stems, *2/5* . The expression level is relatively high in the roots and stems.

Discuss

Plant NOX NOx plays a vital role in plant growth, development, and abiotic stress. To date, NOx... Gene families have been identified and functionally studied in multiple species, including Arabidopsis thaliana, rice, wheat, sweet orange, grape, pigeon pea, cassava, and strawberry [26-28] . However, the tea plant... NOX Gene family studies are scarce. With the development of sequencing technology, genome sequencing of different tea varieties has been completed successively. Therefore, utilizing bioinformatics to study tea varieties... NOX Whole-genome identification and expression analysis of gene families are necessary. Studies have found nine NOX genes in the tea plant genome, a number similar to that of Arabidopsis thaliana and rice, but fewer than in legumes such as soybean. This may be due to… NOX The number of gene families increases with the expansion of the entire legume genome (Table 1). Motif analysis shows that each NADPH gene family has the same motif, indicating high conservation of NOX protein sequences in tea (Figure 4). Furthermore, protein domain and gene structure analysis shows that the number of introns and exons in tea NOX is not significantly different, and domain predictions also show high consistency. Although CsNOX6/9 lacks two EF-Hand domains for binding Ca^{2+} , SMART prediction still found two low-confidence EF-Hand domains at the C-terminus of the NADPH domain. Gene structure and protein domain analysis further demonstrate the high conservation of the tea NOX gene sequence (Figure 3).

When a plant is stimulated by external signals, NOx located on the plasma membrane... It can change its own activity through conformational changes, thereby rapidly regulating the level of reactive oxygen species in intracellular parts to respond to stress and regulate its own growth and development. In Group-I, *CsNOX6/9* and *AtNOXH /J* have high homology. Previous studies have shown that *AtNOXH/J* is mainly expressed in floral organs and is essential for pollen tube elongation and fertilization [16-19] . Combined with RNA-seq expression analysis, it was found that although *CsNOX6/9* was expressed at low levels in all eight tissues, it was expressed at high levels in floral organs, and was also expressed in roots and stems (Figures

1 and 7) . Therefore, *CsNOX6/9* may have similar functions to *AtNOXH /J* , while also playing other roles in roots and stems. In Groups II and III, *CsNOX2* and *AtNOXE* , *CsNOX3/4/5* and *AtNOXI /F* , and *CsNOX1/7/8* and *AtNOXA /C/D/G* showed high homology. Previous studies have found that *AtNOXC /D/F* are involved in the salt stress response [29,30] . RNA-seq expression analysis revealed that *CsNOX1/7* was indeed upregulated in the early stages of salt stress, but downregulated at 72 hours of salt stress. CsNOX5 was downregulated at 24 hours of salt treatment, upregulated at 48 hours, and downregulated at 72 hours. *CsNOX3* expression gradually increased with treatment time, while *CsNOX4 was downregulated at 24 hours of salt treatment, with little change at 48 and 72 hours (Figures 1 and 6). These results indicate that CsNOX1* /7 exhibit significant spatiotemporal characteristics in response to salt stress. It is expressed in the roots, produces ROS, and controls root hair growth [13] . RNA-seq expression analysis found that *CsNOX1/7* are highly expressed in the roots. *AtNOXD,* as a putative housekeeping gene in Arabidopsis, does not show significant differences in expression across different tissues, which is similar to the expression pattern of CsNOX1 [3] (Figure 1, Figure 7) . Therefore, *CsNOX1* may have similar functions to *AtNOXD* , participating in plant stomatal closure, pathogen stress, mechanical damage response, and signal transduction [4] , and together with *CsNOX7 ,* it participates in controlling the growth of tea tree root hairs.

Collinearity analysis revealed that *CsNOX4/5* and *AtNOXI /F* were collinear, suggesting some functional similarity; however, their specific functions require further investigation (Figure 2). *CsNOX7* and *AtNOXC* were also collinear. Furthermore, *CsNOX7* and *CsNOX1* belong to gene pairs resulting from fragment duplication events, with Ka/Ks values significantly less than 1. This indicates that *CsNOX1/7* underwent intense purifying selection during evolution, further suggesting that *AtNOXC* and *CsNOX1/7* should be functionally similar (Figure 2, Table 2). *CsNOX3/5* and *CsNOX6/9* are also gene pairs resulting from fragment duplication events, with Ks/Ks values similarly significantly less than 1 (Table 2). Promoter element analysis showed that *CsNOX* contains numerous stress-responsive elements as well as hormone-responsive elements (Figure 5). For example, the ABRE element contained in CsNOX1/2/7/8 participates in the response to ABA. The response. In Arabidopsis, maize, and tobacco, ABA can induce corresponding NOx. Gene expression is upregulated [31] .

In summary, the NOX gene family in tea plants plays an important role in stress resistance and growth and development regulation, but its specific biological functions require further research.

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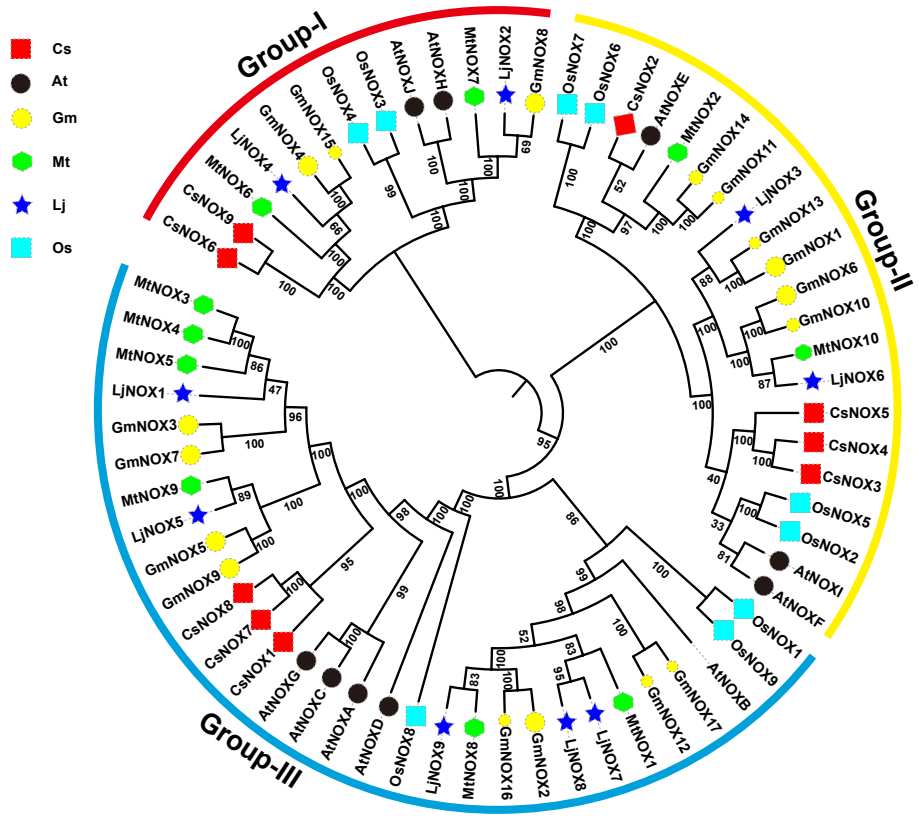

Figure 1. Phylogenetic tree and grouping of *NOX genes* in tea, Arabidopsis thaliana, rice, soybean, alfalfa and birdsfoot .

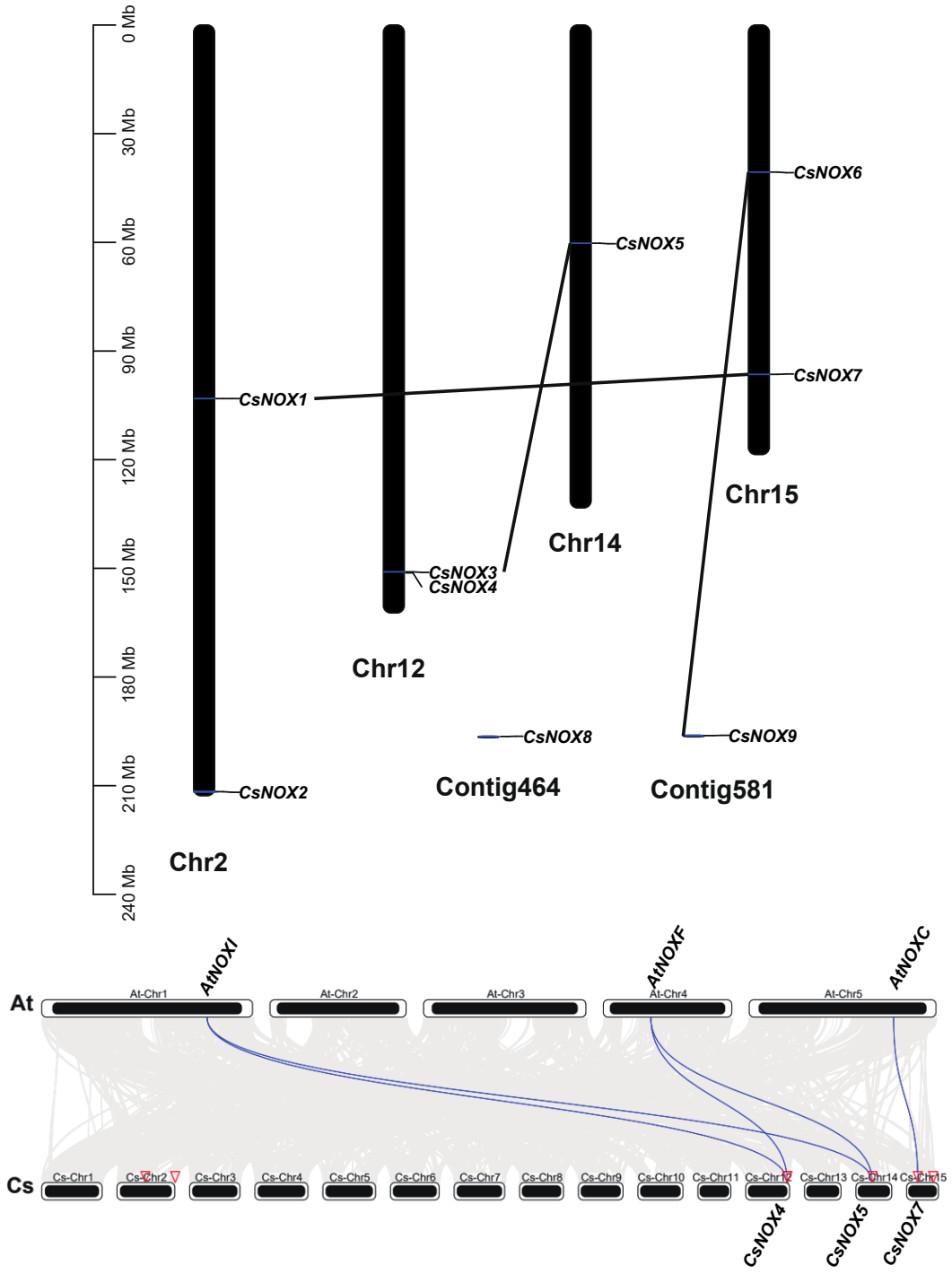

Figure 2. Chromosomal localization of *NOX genes* in tea and collinearity analysis of *NOX genes in tea and Arabidopsis thaliana.*

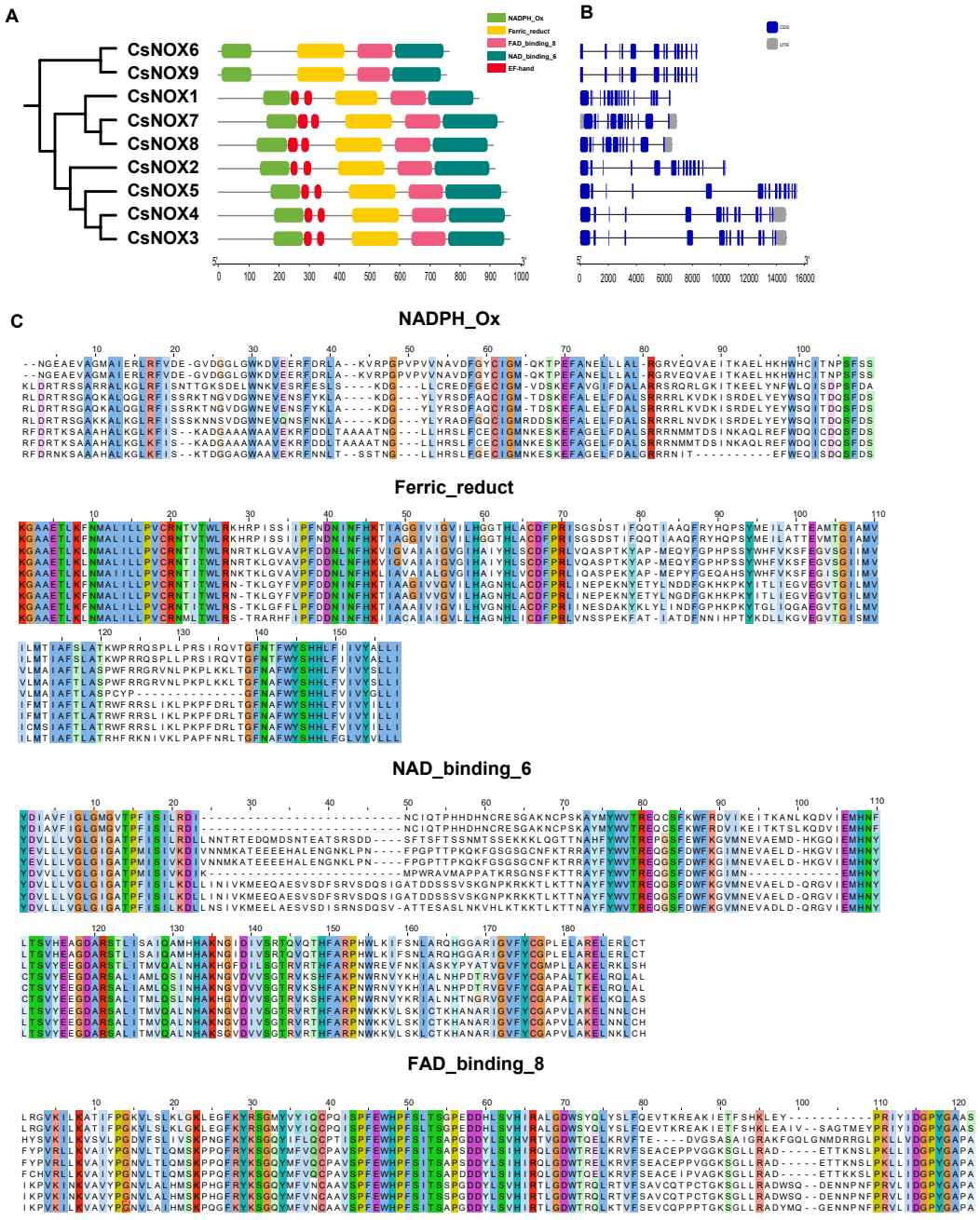

Figure 3. Protein domains (A), intron/exon structures (B), and sequence similarity analysis of the *NOX gene* in tea plants (C).

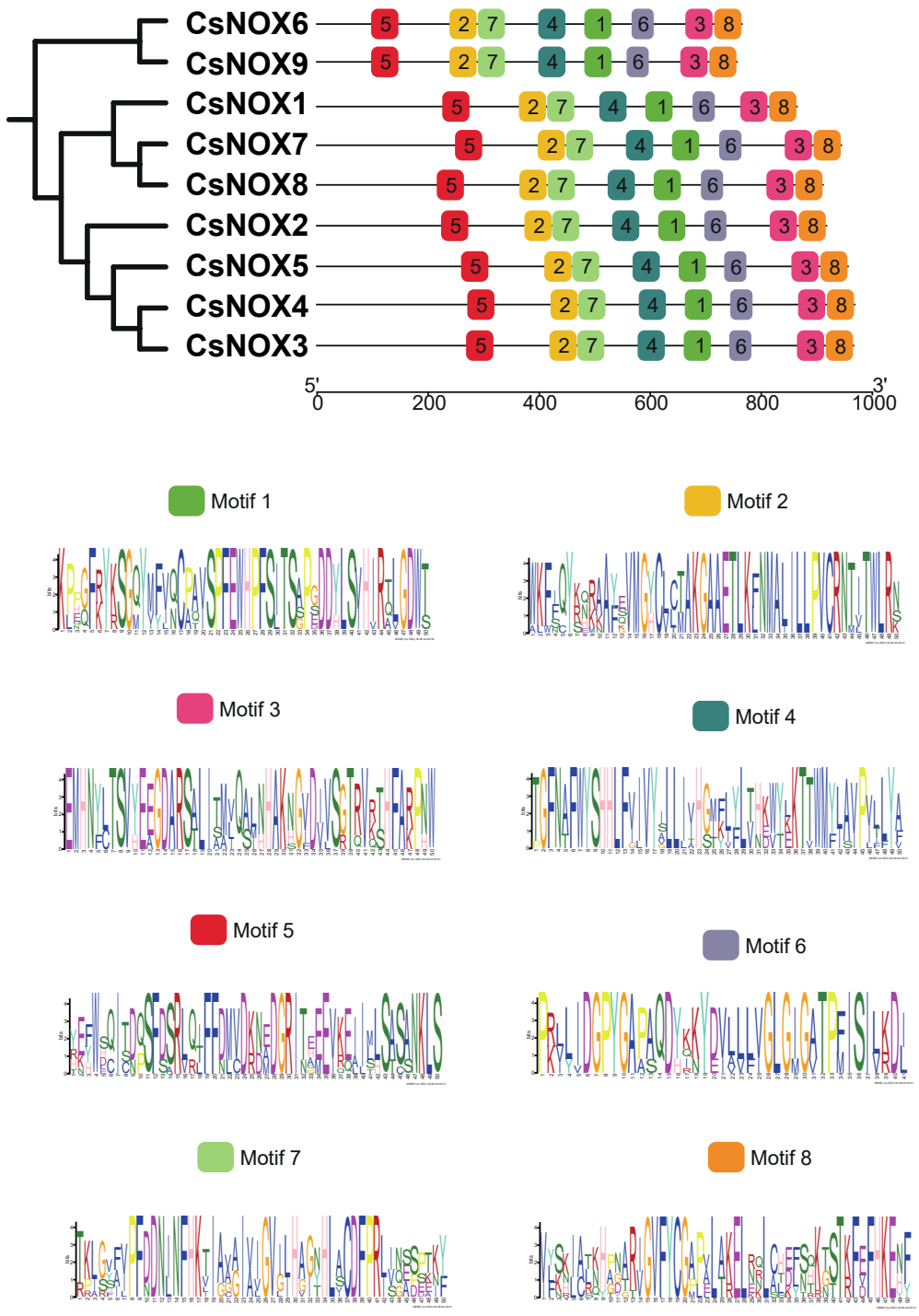

Figure 4. Protein meta- if analysis of NOX from tea plants .

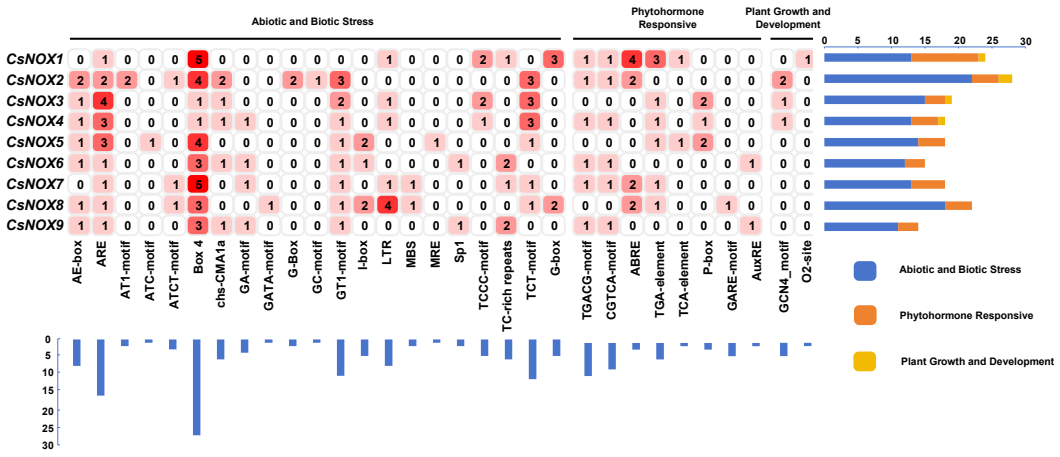

Figure 5. Promoter element analysis of *NOX gene* in tea plant .

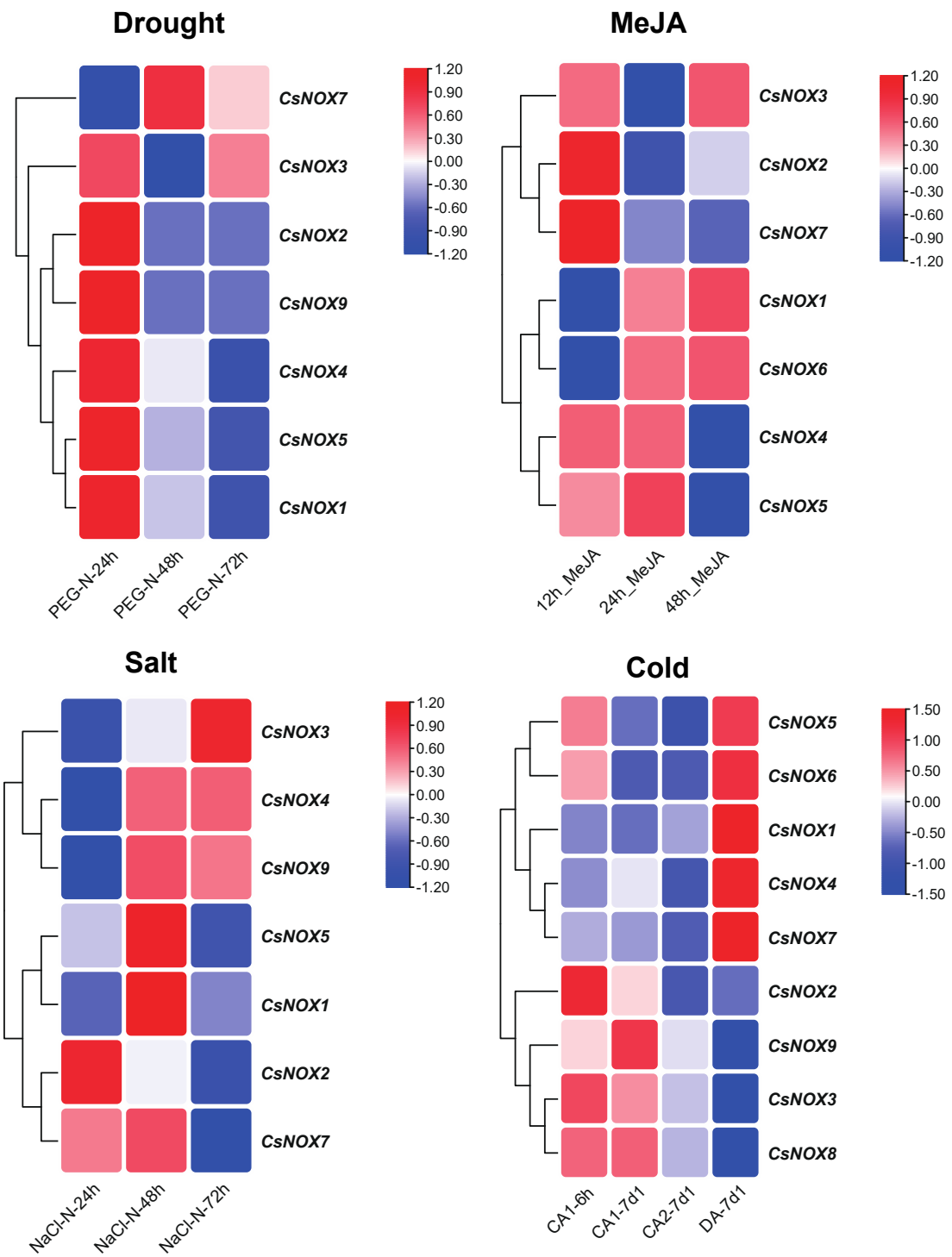

Figure 6. Expression heatmap of *NOX gene in* tea plant under drought, JA , salt and cold stress.

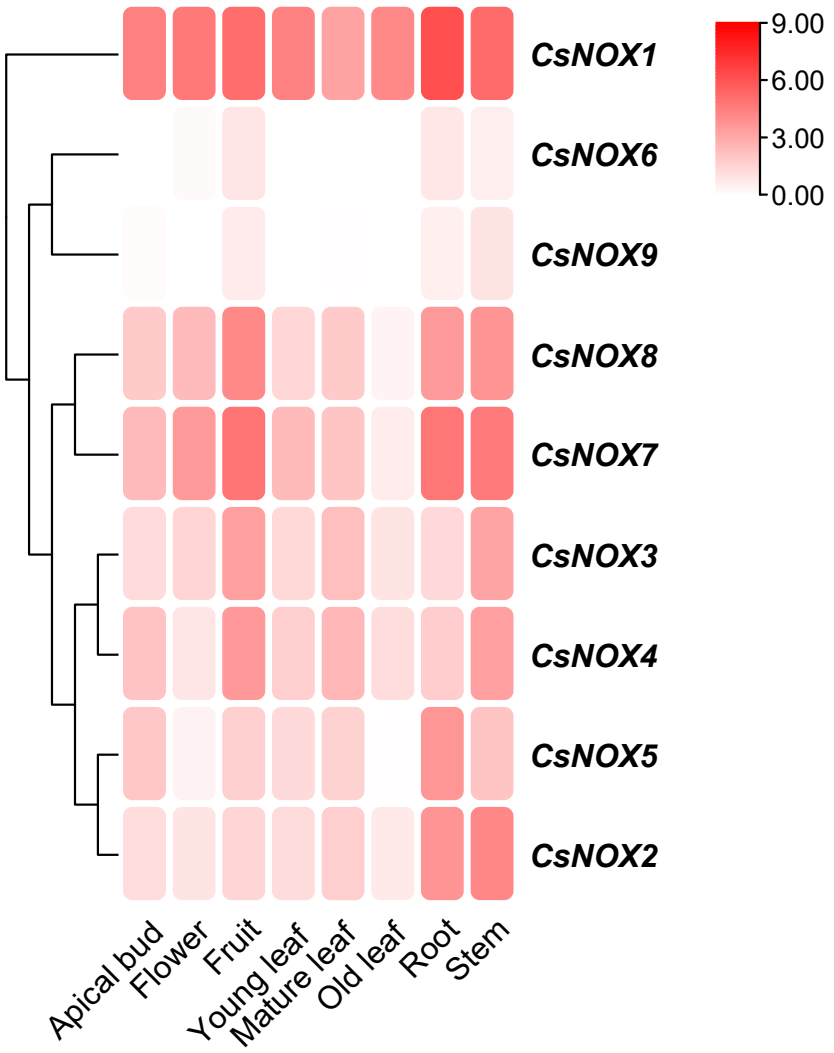

Figure 7. Expression profile analysis of *NOX* gene in important tissues and organs of tea plant .

Table 1: Physicochemical Properties Analysis of NOX Protein

Serial Number	Gene Name	Origin name	Molecular Weight (kDa)	Protein Length (aa)	Pl
1	CsNOX1	CSS0039845	97.01	865	9.39
2	CsNOX2	CSS0046357	104.24	918	8.36
3	CsNOX3	CSS0043661	109.9	967	9.22
4	CsNOX4	CSS0001287	110.11	969	9.19
5	CsNOX5	CSS0047680	108.378	957	9.19
6	CsNOX6	CSS0029939	87.69	766	8.9
7	CsNOX7	CSS0008067	106.4	945	9.11
8	CsNOX8	CSS0045221	102.7	912	9.27
9	CsNOX9	CSS0027498	86.8	757	8.95

Table 2 : Ka /Ks analysis of Cs NOX replication gene

Serial Number	Paralogous Pairs	Ka	Ks	Ka/Ks	Duplication
1	CsNOX3-CsNOX5	0.075158599	0.483189957	0.155546692	SD
2	CsNOX1-CsNOX7	0.099370823	0.723981409	0.137256042	SD
3	CsNOX6CsNOX9	0.001717394	0.003840008	0.447237077	SD

*SD: Segmental Duplication

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