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Research paper

PtKTI12 genes influence wobble uridine modifications and drought stress tolerance in hybrid poplar

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The multisubunit Elongator complex plays key roles in transcription by interacting with RNA polymerase II and chromatin modeling. Kti proteins have been identified as the auxiliary protein for the Elongator complex. However, our knowledge of Kti proteins in woody plants remains limited. In this study, in total 16 *KTI* gene homologs were identified in *Populus trichocarpa*. Among them, the two *KTI12* candidates were named *PtKTI12A* and *PtKTI12B*. Although *PtKTI12A* and *PtKTI12B* were largely different in gene expression level and tissue specificity, both genes were induced by heat and drought stresses. *PtKTI12A* and *PtKTI12B* RNAi transgenic poplar plants showed reduced levels of modified nucleosides, in particular 5-carbamoylmethyluridine and 5-methoxycarbonylmethyl-2-thiouridine. Meanwhile, their tolerance to drought was improved when subjected to withdrawal of watering. Also, the protein products of *PtKTI12A* and *PtKTI12B* had similar subcellular localization and predicted tertiary structure. The results suggest that Kti12 proteins are involved in tRNA wobble uridine modification, stress response and drought stress tolerance in hybrid poplar.

Keywords: drought, KTI12, ncm^{5}U, *Populus*, tRNA.

Introduction

The Elongator complex is a conserved eukaryotic protein complex associated with RNA polymerase II (Otero et al. 1999). The Elongator complex participates in transcriptional elongation as well as chromatin modeling. The holo-elongator complex consists of the core-complex of Elp1–Elp6 and the auxiliary proteins, such as Sit4, Sap185-Sap190 and Kti11-Kti14 proteins (Glatt et al. 2012). One common function of these proteins is wobble uridine modifications at position 34 of certain tRNAs (Table S1 available as Supplementary Data at *Tree Phyisology* Online, Krogan and Greenblatt 2001, Mehlgarten et al. 2010, Versees et al. 2010, Chen et al. 2010). The wobble uridine modifications include mcm^{5}U (5-methoxycarbonylmethyluridine), mcm^{5}s^{2}U (5-methoxycarbonylmethyl-2-thiouridine), ncm^{5}U (5-carbamoylmethyluridine) and ncm^{5}Um (5-carbamoylmethyl-2'-O-methylurdine). The mutants *elp1-6* and *kti11-14* (killer toxin-insensitive) of the yeast *Saccharomyces cerevisiae* are defective in these modifications (Huang et al. 2008). In *S. cerevisiae*, mcm^{5}s^{2}U is present on tRNA-Glu-UUC (glutamine tRNA with UUC as anticodon), tRNA-Gln-UUG and tRNA-Lys-UUU, whereas ncm^{5}U is present on tRNA-Pro-UUG and tRNA-Val-UAC. In the absence of mcm^{5}s^{2}U modification, for example in *kti11-14* mutants, tRNA-Glu, tRNA-Gln and tRNA-Lys could not be recognized by tRNA endonuclease *Kluyveromyces lactis* γ-toxin, rendering a toxin-resistance phenotype (Frohloff et al. 2001; Jablonowski et al. 2001b; Lu et al. 2005). The most important subunit of the elongator core complex is Elp3 protein as a non-canonical acetyltransferase and genuine tRNA modification enzyme (Lin et al. 2019, Walker et al. 2011, Winkler et al. 2002, Wittschieben et al. 1999). The largest subunit of the core complex is Elp1, whose phosphorylation

status is regulated by the cofactors, such as Sit4, Sap185, Sap190 (Jablonowski et al. 2001a; Jablonowski et al. 2004) and Kti11-Kti14 (Mehlgarten et al. 2009, Petrakis et al. 2005). Kti proteins can physically interact with the Elp proteins and regulate their function (Fichtner et al. 2002, Frohloff et al. 2001, Glatt et al. 2012, Kolaj-Robin et al. 2015). For example, Kti12 and Kti14 co-ordinately regulate the phosphorylation status of Elp1 and therefore the core activity of the Elongator complex (Abdel-Fattah et al. 2015, Jablonowski et al. 2004).

KTI12 was one of the first *KTI* (*killer toxin-insensitive*) genes identified from *S. cerevisiae* (Butler et al. 1994, Fichtner et al. 2002, Frohloff et al. 2001, Nelissen et al. 2003). Annotated as chromatin-associated proteins, Kti12 proteins can physically interact with the Elp3 subunit, and are therefore critical for the function of the holo-Elongator complex (Petrakis et al. 2005). The P-loop motif within Kti12p is suggested for ATP (adenosine-triphosphate) binding, and mutation on the critical residues within the P-loop leads to the absence of wobble uridine modification and zymocin resistance (Mehlgarten et al. 2017). Serial deletion of the calmodulin-binding-domain (CBD) on the C-terminal of ScKti12 leads to protein dysfunction and zymocin resistance, suggesting CBD is important for Kti12 function (Mehlgarten et al. 2017). A previous study showed that subunits of Elongator complex from yeast and plants could ectopically complement each other (Mehlgarten et al. 2010). The *Arabidopsis thaliana kti12/drl1* mutant showed disorganized shoot and root meristems (Cho et al. 2007, Nelissen et al. 2003), whereas *elp* mutants showed pleiotropic phenotypes including reduced organ growth, hormone, elevated drought stress tolerance, oxidative stress supersensitivity, and anthocyanin accumulation (Defraia et al. 2013, Nelissen et al. 2005, 2010, Wang et al. 2013, Xu et al. 2012, Zhou et al. 2009). The *Arabidopsis* Kti12 homolog AtDRL1 partially complements the *S. cerevisiae* mutant for growth retardation, but not as whole functional exchange (Jun et al. 2015). Shuffling of the P-loop or CBD motif between AtDRL1 and Kti12p also suggests that plant Kti12 proteins have undertaken sequence and function diversification (Mehlgarten et al. 2017).

Modified nucleosides around the anticodon loop region are of importance for the decoding process. ncm^5U, mcm^5U and mcm^5s^2U are complicated modifications present only at the wobble position. ncm^5U has been comprehensively identified at tRNAs from bacteria and eukaryotes, including yeast, *Arabidopsis* and calf liver, but not in archaea. The modification pathway of ncm^5U and structurally related mcm^5U has not been completely resolved. At least 14 proteins were shown to be involved in the 5′-side chain modification in *S. cerevisiae* (Huang et al. 2008), including Elp1-6, Kti11-14, Trm9, Sap185 and Sap190. Mutations in any of these genes led to deficiency in wobble uridine modifications and killer toxin-resistance phenotype in *S. cerevisiae*. Multiple studies indicate that there are connections between these three wobble uridine nucleosides and members of the Elongator complex. The function of Kti proteins are associated with this process due to their function involved in regulating the phosphorylation status of Elp1 and therefore the stability of the whole Elongator complex. Kti14 kinase activity requires Kti11, Kti12 and Kti13, therefore null-mutant of *KTI* genes would be anticipated to have wobble uridine deficiency as well. Other phenotypes resulting from defects in wobble uridine modification most likely arise from a compromised translation by hypo-modified tRNAs.

The connection of the Elongator complex and tRNA wobble uridine modification has been well established; one of the other physiological functions in plants concerns their regulatory role in plant stress tolerance. Indeed, many of the *elp* mutants show developmental phenotypes or abberent stress response, including both abiotic and biotic stresses (Chen et al. 2006; Defraia et al. 2013, DeFraia et al. 2010, Kojima et al. 2011, Wang et al. 2013, Xu et al. 2012, Zhou et al. 2009). We reviewed some of the work on plant Elp and Kti proteins in 2013 (Yan et al. 2013), Kti12 shares structural similarity with the O-phosphoryl-tRNA kinase (PSTK) and contains a P-loop motif for ATP/GTP binding (Krutyhołowa et al. 2019). The narrow-leave phenotype of *kti12/drl1* in *Arabidopsis* is very similar to *elp1*, *elp2*, *elp4* and *elp6* mutants (Nelissen 2003, 2005), and the *elp1* mutant is more drought tolerant (Chen et al. 2006). DeFraia and Mou reviewed the role of the Elongator complex in a plant (DeFraia and Mou 2011) and recent work showed the elongator's function in plant meristem patterning and immune response (Jun et al. 2015, Nakai et al. 2019, Wang et al. 2013, 2018, Xu et al. 2012, Zhu et al. 2015). Hundreds of transcripts were affected in the *Arabidopsis drl1/kti12* mutant, in particular those associated with defense response or wound response, suggesting a potential regulatory module from the elongator via tRNA modification on protein/gene expression to influence stress and immune response (Wang et al. 2018).

This study aims to study the function of Kti12 protein in poplar, both for its involvement in tRNA wobble uridine modification, and more importantly whether a change in *PtKTI12* activity could lead to physiological changes relevant for stress tolerance. We used bioinformatics, qRT-PCR, protein localization and structure modeling, and genetic manipulation of *PtKTI12* genes by RNAi to investigate these issues. Our data suggested that *KTI12* genes in *Populus* are important for wobble uridine modification and stress tolerance under water deficit conditions. Our results fill the current blank of tRNA modification in woody plants and suggest a new strategy to breed drought-resistant poplar.

Material and methods

Plant material and growth conditions

Hybrid poplar *P. tremula* × *alba* 717-1B4 was propagated by tissue culture on MS medium supplemented with 0.05 mg/l

thidiazuron (TDZ) at 25° C, with light intensity at 150 µmol m^{-2} s^{-1}, 16/8 h photoperiod. Plantlets were grown in glass bottles in the culture room until 10 cm in height, then were transferred to soil to grow in a greenhouse with similar settings of irradiation and photoperiod. The relative humidity in the greenhouse was 60–70%.

Populus transformation

Primers were designed corresponding to the 3′-end of the cDNA sequence of *PtKTI12A* or *PtKTI12B* (Table S1 available as Supplementary Data at *Tree Phyisology* Online). Amplified RNAi fragments were cloned via entry vector *pGWC* (Chen et al. 2006) into gateway vector *pH7GWIWG2(II)* (Karimi et al. 2002) by LRase mediated recombination using Gateway technology. *Agrobacterium* strain C58 (Rif^R) was used for poplar transformation by the co-cultivation method (Leple et al. 1992) with hygromycin as a selection marker. Positive seedlings were tested by qRT-PCR for *PtKTI12A* or *PtKTI12B* relative gene expression. Verified transgenic lines were propagated by tissue culture to 10 cm high before being transferred to pots and grown in a greenhouse for further experiments.

Bioinformatics analysis of PtKTI genes

Kti12p of *S. cerevisiae* was used as query sequence for retrieval of plant homologs using blastp search with cutoff value at 1.0e-6. cDNA and CDS sequences of *PtKTI12A* and *PtKTI12B* were downloaded from Phytozome (http://www.phytozome.net) (Goodstein et al. 2012). Protein domain structure was predicted by pfam (http://pfam.xfam.org/) (Finn et al. 2014). Protein multisequence alignment was performed by ClustawX, a non-rooted neighborhood joining tree was constructed using MEGA5.0 (Tamura et al. 2007). The bootstrap analysis was performed with 1000 iterations.

Expression profiling of PtKTI12A and PtKTI12B genes

Populus microarray or RNAseq data were downloaded from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.gov/gds/), with accession numbers as following: GSE97463 (drought); GSE21480 (cold); GSE124805 (heat); GSE16785 (wounding); GSE19297 (aluminium); GSE60311 (salt); GSE16773 (methyl jasmonate treatment); GSE9673 (pathogen infection) and GSE14894 (nitrogen limitation). *PtKTI12A* and *PtKTI12B* tissue-specific expression profiles were extracted from PopGenIE (http://popgenie.org/exheatmap) (Sjödin et al. 2009). The genes expression level before stress conditions was defined as A_x, and the genes expression level after stress conditions as B_x. We defined the proportion of changes in this gene affected by stress as C_x. The function is expressed as follows:

$$C_x = \frac{B_x - A_x}{A_x}$$

The C_x value represents the impact of stress on plants. Gene expression data for *PtKTI12B* could not be found in GEO database, maybe due to its overall low expression.

Wobble uridine nucleoside analysis by LC-MS/MS

Small RNAs were extracted using microRNA Extraction Kit (Omega Bio-tek Inc.). 20 µg tRNA was digested with P1 nuclease (Sigma) and Calf intestine alkaline phosphatase (Toyobo), diluted with Milli-Q water to 10 µg/ml before injected (Wang et al. 2017). The injection volume was 10 µl. An Inertsil ODS-3 column (2.1 × 150 mm, 5 µm particle size; Shimadzu) was used for nucleoside separation with gradient described previously (Wang et al. 2017). Multi-reaction monitoring mode was used with a mass scan from 100 to 700 Da on an API 4000 Q-Trap mass spectrometer with parameters according to Jin et al. (2019).

Determination of poplar growth curve and photosynthetic rates

Poplar was recorded for its initial height before transferring from tissue culture to pot for growth curve measurement with 7 days interval for 5 weeks. Photosynthetic rate was determined on 2-month-old plants by Li-6400XT portable photosynthesis system (LiCor Biosciences Inc., Lincoln, Nebraska, USA), with instrument settings according to Lawrence et al. (2019) and Xin et al. (2019).

Application of heat and drought stress on hybrid poplar

Drought stress treatment was performed on 3-month-old seedlings; heat stress was applied by transferring plants from greenhouse to a growth chamber set to 37° C or 42° C, with similar photoperiod and light conditions. For drought stress, poplar leaf samples were taken for qRT-PCR at 0, 3, 6, 9, 11, 13 and 14 d, and for morphological and physiological assays, leaf samples were taken 5 days after drought stress. For heat stress, leaf samples were taken in triplicates at time-points of 0, 0.5, 1, 2, 4, 8, 12, 24 and 48 h. All experiments were performed with three biological replicates at each time-point. Samples were flash-frozen by liquid nitrogen and stored at −80° C until further use.

Quantitative RT-PCR

Total RNA was extracted by Trizol reagent (Tiangen Biotech, Beijing, China) and reverse transcribed to cDNA using EasyScript One-Step gDNA Removal and cDNA Synthesis SuperMiX (TransGen Biotech, Beijing, China). qRT-PCR was conducted using a BioRad IQ5 real-time PCR system (Life Science, Wuhan, China), with *PtUBQ4* (POPTR_0001s44440) as the reference gene.

Measurements of total chlorophyll, proline and malondialdehyde contents

Leaf samples were taken from 1.0 m-height 717 hybrid poplar (control) and *PtKTI12A* or *PtKTI12B* RNAi transgenic plants,

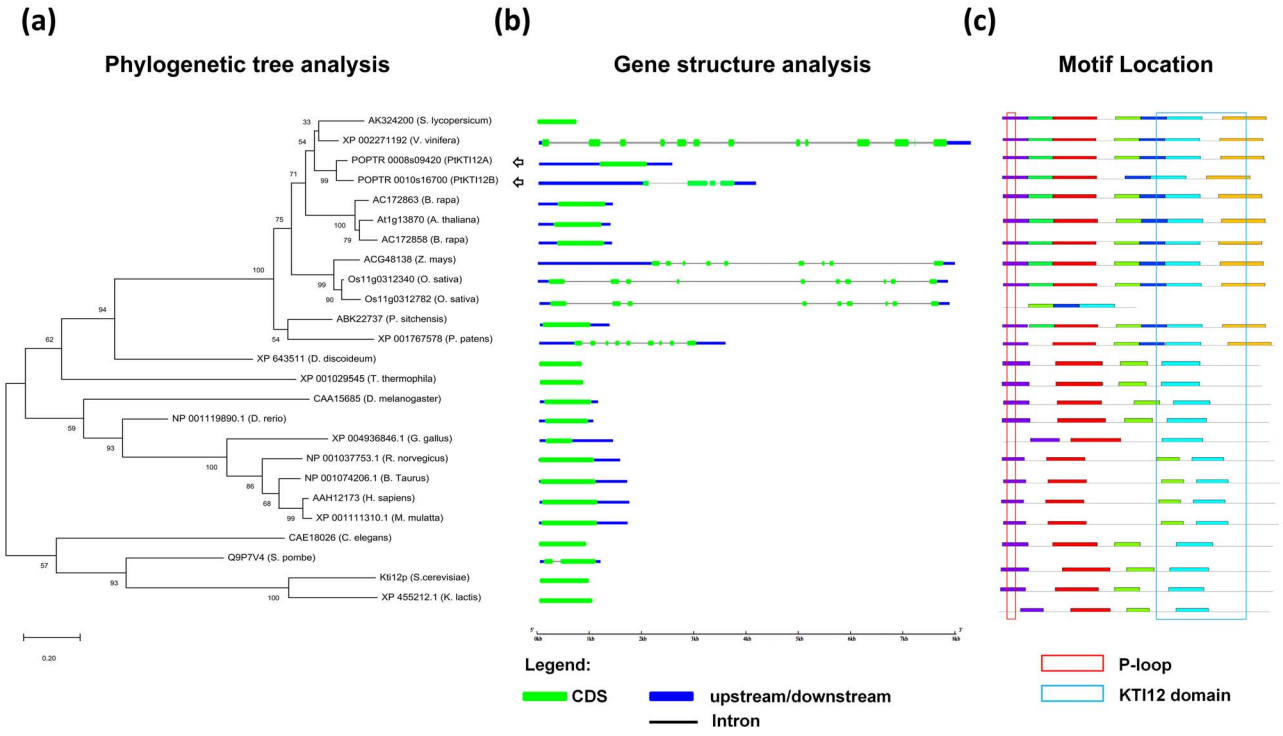

Figure 1. Phylogenetic analysis of Kti12 homologs in plants. (a) NJ tree generated by MEGA5 software using full protein sequences. Numbers in each branch indicate bootstrap analysis supporting value. PtKTI12A and PtKTI12B are indicated by arrows to the right. (b) Gene structure of Kti12 homologs. Blue lines indicate 5' UTR or 3' UTR; green boxes indicate exons; gray lines indicate introns. Scale bar underneath indicates region length in kilobases (kb). (c) Motif analysis of Kti12 homologs. Motifs were predicted by MEME motif analysis online (http://meme-suite.org/tools/meme), with 10 as the maximum number of motifs for each protein. Motifs were displayed in color mode; the region for P-loop and Kti12 domain are roughly indicated with red and blue boxes, respectively.

before (time zero) or 5 days after drought treatment. Total chlorophyll content was measured according to Inskeep and Bloom (1985). As previously described, proline and malondialdehyde contents were determined using the leaf samples of 5 days after drought stress (Jing et al. 2016).

Protein subcellular localization and tertiary structure prediction

A *pD1301s-eGFP* vector was used with eGFP tag fused to the C-terminus of PtKti12A or PtKti12B proteins. The resulting constructs were transformed into *Agrobacterium* strain GV3101, and tobacco leaves transient infiltration was used for protein subcellular localization (Sparkes et al. 2006). Chloroplast autofluorescence and an ER mCherry-HDEL marker were used to locate subcellular compartments. Fluorescent signals were visualized using a confocal laser scanning microscope (Leica SP5 CLSM) with ×63 objective lens. *pD1301s-eGFP* empty vector serves as a control.

The protein sequence of CtKTI12 (*Chaetomium thermophilum*) with known structure was downloaded from PDBe (Krutyholowa et al. 2019). PtKTI12A and PtKTI12B protein sequences were downloaded from the Phytozome website (https://phytozome.jgi.doe.gov/pz/portal.html#). Protein tertiary structure prediction was generated by Phyre2 online (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) (Kelley et al. 2015) and visualized by PyMOL-2.3.2 software (https://pymol.org/2/).

Results

Expression of KTI12 homologs was induced by drought and heat stresses in Populus

With protein sequence homology, we identified two Kti12 homologs, named as PtKTI12A (POPTR_0008s09420) and PtKTI12B (POPTR_0010s16700), with a cutoff value of 1.0E-06 (Figure 1a). Unrooted NJ-tree of Kti12 homologs revealed a close relationship between *Populus* Kti proteins with those from grape (*Vitis vinifera*), rapeseed (*Brassica rapa*) and *A. thaliana* (Figure 1a). The group of monocot Kti proteins separated from the dicot group, meanwhile both groups had distant relationships with the Kti homologs from bacteria, yeast, *C. elegans* and animals (Figure 1a). As for gene structure, *PtKTI12A* had a single exon, whereas *PtKTI12B* had four exons (Figure 1b); their lengths of CDS were 909 bp and 879 bp, respectively (Table S2 available as Supplementary Data at *Tree Physiology* Online). All Kti homologs had similar motif organization, containing a single Kti12 domain in each protein and a P-loop motif in most of the members (Figure 1c,

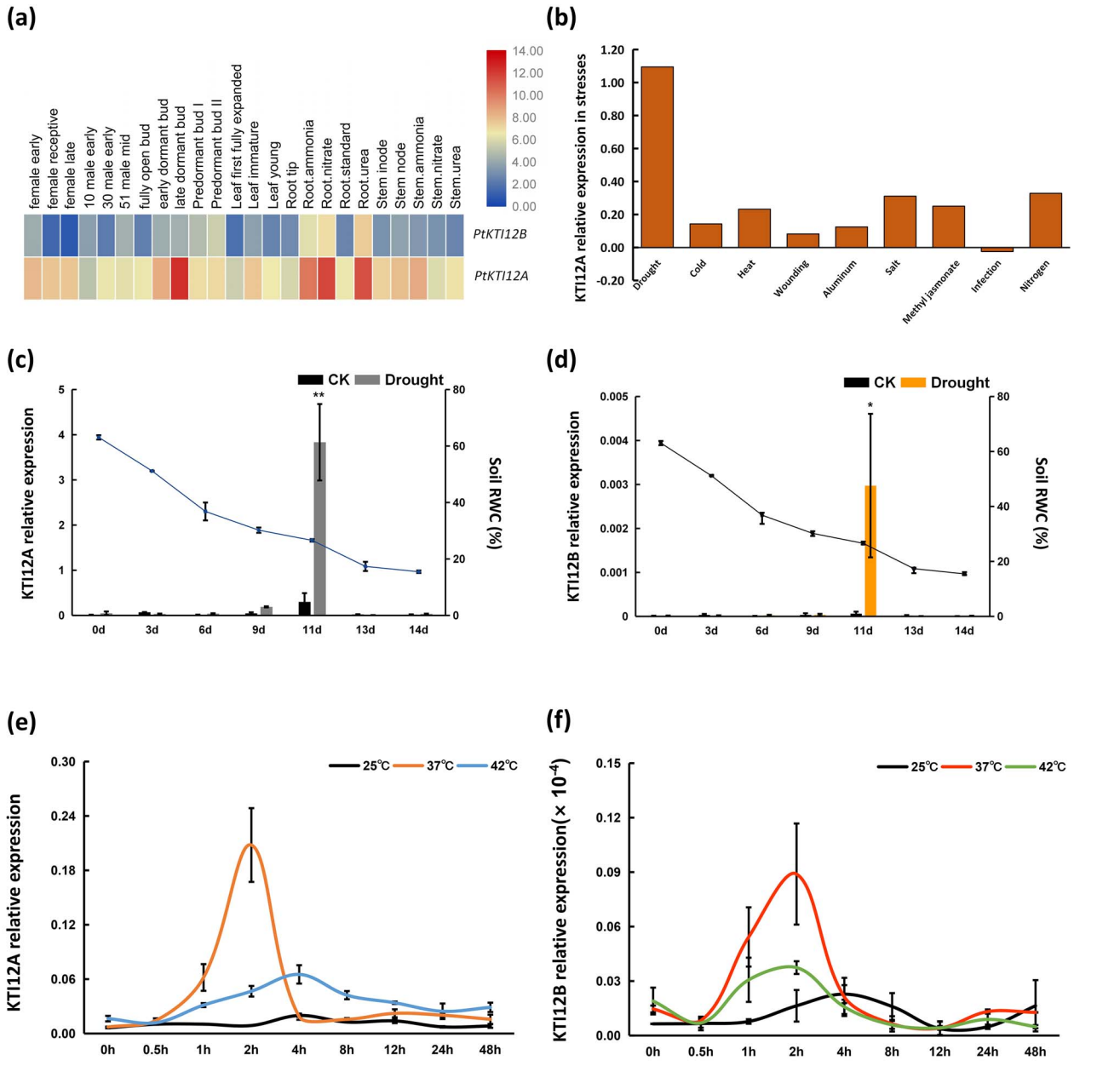

Figure 2. Expression analysis of *PtKTI12A* and *PtKTI12B* in tissues and upon stress conditions. (a) Heat map generated using expression data from Phytozome. (b) *PtKTI12A* relative expression upon various stress conditions, GSE97463 (drought); GSE21480 (cold); GSE124805 (heat); GSE16785 (wounding); GSE19297 (aluminium); GSE60311 (salt); GSE16773 (methyl jasmonate); GSE9673 (pathogen infection); GSE14894 (nitrogen limitation). (c) and (d) qRT-PCR analysis of *PtKTI12A* and *PtKTI12B* transcript levels upon drought stress/desiccation treatment. Bar to the right indicates relative water content (RWC) in soil; bar to the left indicates gene relative expression. * and ** significant at $P < 0.05$ and $P < 0.01$, respectively, by Student's t-test. (e) and (f) Gene relative expression of *PtKTI12A* and *PtKTI12B*, respectively, under heat stress. Data from three biological replicates for each time-point.

Figure S1 available as Supplementary Data at *Tree Physiology* Online). The overall protein sequence similarity between PtKti12A and PtKti12B was 62.3%, yet there was a deletion of 30 amino acids within the Kti12 domain in PtKti12B (Figure S1 available as Supplementary Data at *Tree Physiology* Online). Microarray data downloaded from PopGenIE (http://popgenie.org/) suggested that *PtKTI12A* had a higher transcript level than *PtKTI12B* in most of the tissues examined, especially in dormant bud and root samples (Figure 2a). The *Populus* eFP visualization tool (http://bbc.botany.utoronto.ca/efppop/cgi-bin/efpWeb.cgi) also revealed differential tissue distribution of *PtKTI12A* and *PtKTI12B* transcripts, with higher *PtKTI12A* expression in mature leaves, but lower in female catkins, suckers, young leaves and stem cross-sections (Figure S2 available as Supplementary Data at *Tree Physiology* Online). GEO expression data suggested *PtKTI12A* expression was strongly induced by

drought and heat stress, moderately by salt, nitrogen limitation and methyl jasmonate treatment (Figure 2b). Our qRT-PCR experiment confirmed that both *PtKTI12A* and *PtKTI12B* could be up-regulated by drought or heat stress (Figure 2c–f). Both genes were strongly up-regulated at the 11th day after drought stress treatment (Figure 2c and d), while their expression levels peaked at the time-point of 2 h after heat treatment at 37° C (Figure 2e and f). The two genes showed similar expression patterns in both stress treatments, except that the expression level of *PtKTI12B* was in general much lower than that of *PtKTI12A*.

Down-regulation of PtKTI12A or PtKTI12B reduced the level of wobble uridine modifications

The strong up-regulation of *PtKTI12A* and *PtKTI12B* during drought stress suggested their possible function during drought response and tolerance. We took a RNAi approach in 717 hybrid poplar to study the function of *PtKTI12A* and *PtKTI12B* on wobble uridine modification, development and stress tolerance in woody species. About 20 independent hygromycin-resistant transgenic lines of each gene were selected and further analyzed by qPCR. Down-regulation of the transcript levels of *PtKTI12A* and *PtKTI12B* was confirmed in the corresponding RNAi lines (Ai for *PtKTI12A* RNAi and Bi for *PtKTI12B* RNAi) ranging from 7–25% to 10–31%, respectively, compared with the control plants (Figure 3a). Three representative RNAi lines for each gene were used for propagation and subsequent analysis.

Total small RNA was extracted and the levels of wobble uridine nucleosides, including ncm^{5}U and mcm^{5}s^{2}U, were determined by LC-MS (mcm^{5}U was not included for technical reasons). The abundance of wobble uridine nucleosides was reduced to different extents, with the most reduction in transgenic lines Ai-16, Ai-9 and Bi-14 (Figure 3b). Approximately 16% of ncm^{5}U and 13% of mcm^{5}s^{2}U were retained in Ai-16 transgenic plants, compared to control plant 717 (Figure 3b). Figure 3c shows the chromatogram of ncm^{5}U and mcm^{5}s^{2}U by LC-MS/MS, together with the four canonical nucleosides (cytidine, uridine, guanosine and adenosine), which are used to quantify the relative amount of each modified nucleoside (Figure 3c). We can see that the endogenous levels of ncm^{5}U and mcm^{5}s^{2}U were rather low, with signal intensity approximately 1.0E+04 (Figure 3c); the signal for mcm^{5}U was even lower (less than 1.0 + E03), therefore making it difficult to quantify with the current method.

PtKTI12A and PtKTI12B RNAi plants showed higher drought stress tolerance

The growth rate of *PtKTI12A/B* RNAi transgenic plants was compared with the control plants during the juvenile growth stage (Figure S3 available as Supplementary Data at *Tree Physiology* Online). Under normal greenhouse conditions, no significant changes in plant height could be observed in transgenic plants, neither in plant architecture, leaf morphology or photosynthetic rate (Figure 4a and b, Figure S3 available as Supplementary Data at *Tree Physiology* Online). This result suggests that although the down-regulation of *PtKTI12A* or *PtKTI12B* influenced the level of wobble uridine nucleosides, it did not influence plant growth under normal conditions.

A previous study showed that an *Arabidopsis atelp1* mutant defective oin ncm^{5}U nucleoside was more tolerant to drought (Chen Z et al. 2006, Zhou et al. 2009). Also in line with their up-regulation upon drought treatment, PtKti12A and PtKti12B are potentially involved in the responses to drought stress. To test this hypothesis, a drought/water withholding experiment was performed on whole plants of *PtKTI12A* and *PtKTI12B* RNAi lines and control plants, recording plant morphology and physiological parameters before and after drought stress (Figures 4 and 5). After 5 days of drought treatment, both *PtKTI12A* and *PtKTI12B* RNAi plants exhibited better morphology than the control plants (Figure 4b and e). The RWC was significantly higher in Ai-21, Ai-31 and especially Bi-14 and Bi-15 lines compared to their control at the end of drought treatment (Figure 4c and f).

We measured chlorophyll contents, MDA (malondialdehyde) and proline leakage as an indicator of cell destruction. Drought stress led to increased chlorophyll content in both control and transgenic plants, but the increased percentage in *PtKTI12A* and *PtKTI12B* RNAi plants was significantly less than that of 717 control plants (Figure 5a and b). More importantly, as proline and MDA are indicators of plants tolerance to stress, the percentage increment of proline and MDA content was also less in RNAi lines compared to the control, indicating less cell membrane damage (Figure 5c–f). Taken together, these data suggest the down-regulation of *PtKTI12A* or *PtKTI12B* led to better drought tolerance in hybrid poplar.

PtKti12A/B protein shared a similar subcellular localization and structure prediction model

The yeast Kti12 protein has been shown to be physically interacting with the core Elongator complex, especially Elp3 (Petrakis et al. 2005). Therefore, as a member of the eukaryotic transcription elongation complex, Kti12 proteins are expected to reside in the nucleus. In order to test this, we constructed eGFP fusion proteins for PtKti12A and PtKti12B. The eGFP tag was fused to the C-terminus of PtKti12A or PtKti12B proteins, respectively. Subcellular localization was examined by tobacco leaf epidermis cell transcient transfection (Figure 6). Chlorophyll autofluorescence and an ER-marker protein (mCherry-HDEL) were used to locate subcellular compartments. For PtKti12A-eGFP and PtKti12B-eGFP constructs, we observed GFP signals mostly in the cytoplasm, and also in the nucleus (Figure 6a–d and i–l). There was weak signal in chloroplast for PtKti12A-eGFP (Figure 6c),

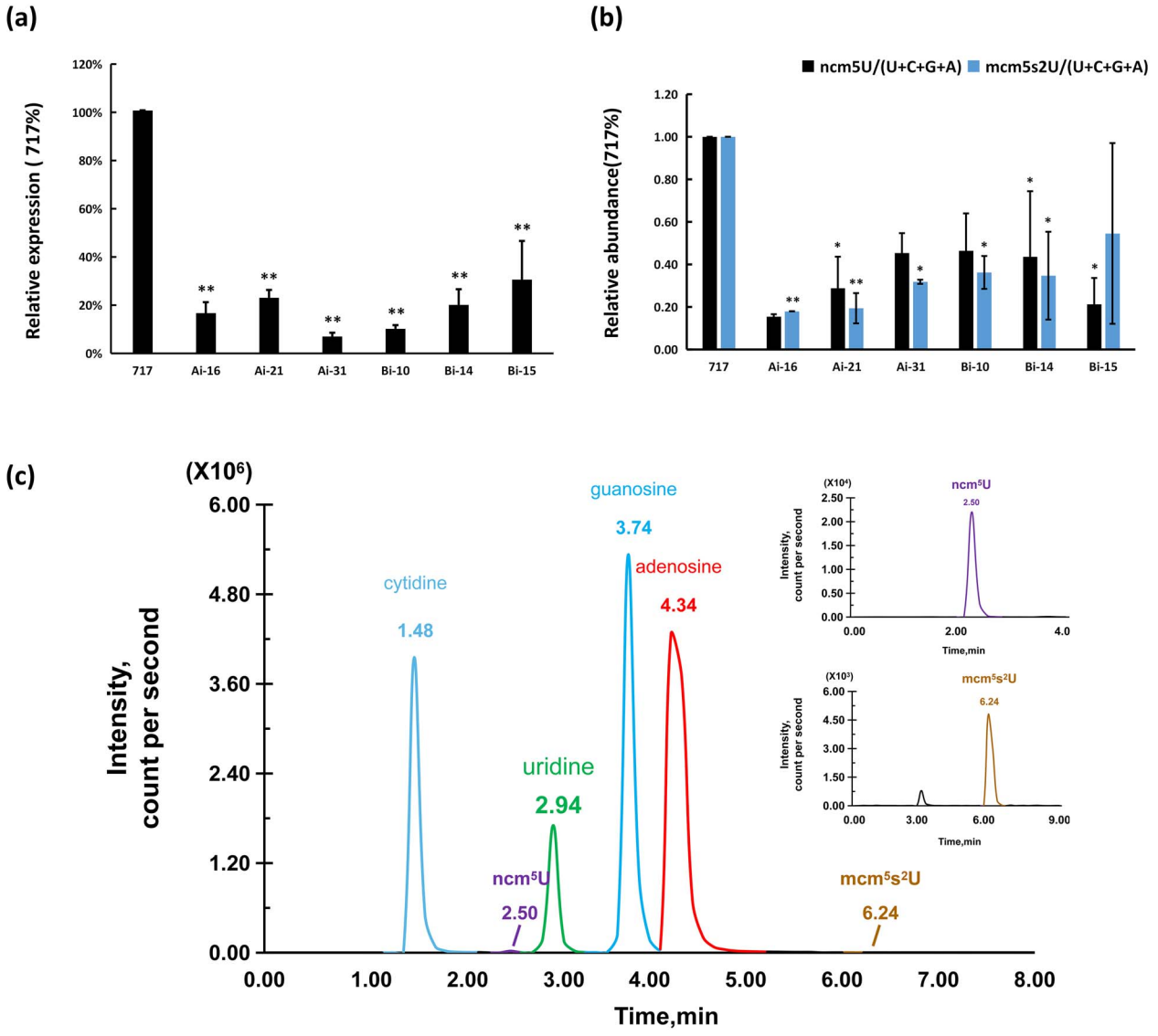

Figure 3. Down-regulation of *PtKTI12A* or *PtKTI12B* led to the reduction of wobble uridine modifications. (a) Gene relative expression in RNAi transgenic plants as compared to poplar 717 control. Standard deviation (SD) was calculated from data from three biological replicates, ∗ and ∗∗ significant at $P < 0.05$ and $P < 0.01$, respectively, by Student's t-test. (b) Quantification of wobble uridine modifications in RNAi transgenic plants. Relative abundance in poplar 717 control plants was artificially set to 1.00. (c) LC-MS chromatogram of nucleosides quantified in this study. The X-axis indicates retention time (RT) in minutes, and the Y-axis indicates signal intensity. Numbers above each peak indicate retention time for each unique nucleoside: Cytidine (RT = 1.48 min), uridine (RT = 2.94 min), guanosine (RT = 3.74 min), adenosine (RT = 4.34 min), ncm^{5}U (RT = 2.50 min) and mcm^{5}s^{2}U (RT = 6.24 min). Quantification of nucleoside was performed based on peak area.

but no signal in ER for PtKti12B-eGFP (Figure 6o). The signal distribution in both cases was rather similar to that for the p35S-GFP construct, which resided both in the cytoplasm and in the nucleus (Figure 6e–h and m–p). Therefore, we conclude that PtKti12A and PtKti12B proteins were localized both in the cytoplasm and nucleus.

Recently, Krutyholowa et al. (2019) resolved the crystal structure of the Kti12 N-terminal nucleotide hydrolase domain from *Chaetomium thermophilum* at 2.4 Å resolution. The NTD structure of CtKti12 in the transition state of ATP hydrolysis strikingly resembles that of PSTK, an O-phosphoseryl-tRNA kinase in archaea required for the synthesis of tRNASec (Krutyholowa et al. 2019). The P-loop present in all Kti12 proteins is suggested for ATP binding and hydrolysis, which is required for presenting the tRNA substrate to the Elongator complex. In line with this, we performed protein tertiary structure prediction on PtKti12A, PtKti12B and CtKti12 (Figure 7). We found that the overall structure of the three proteins were rather similar, including all α-helixes, β-strands and loop regions (Figure 7d). The overall tertiary structure similarity of PtKti12A and PtKti12B with CtKti12 support their role in substrate tRNA binding and wobble uridine modification.

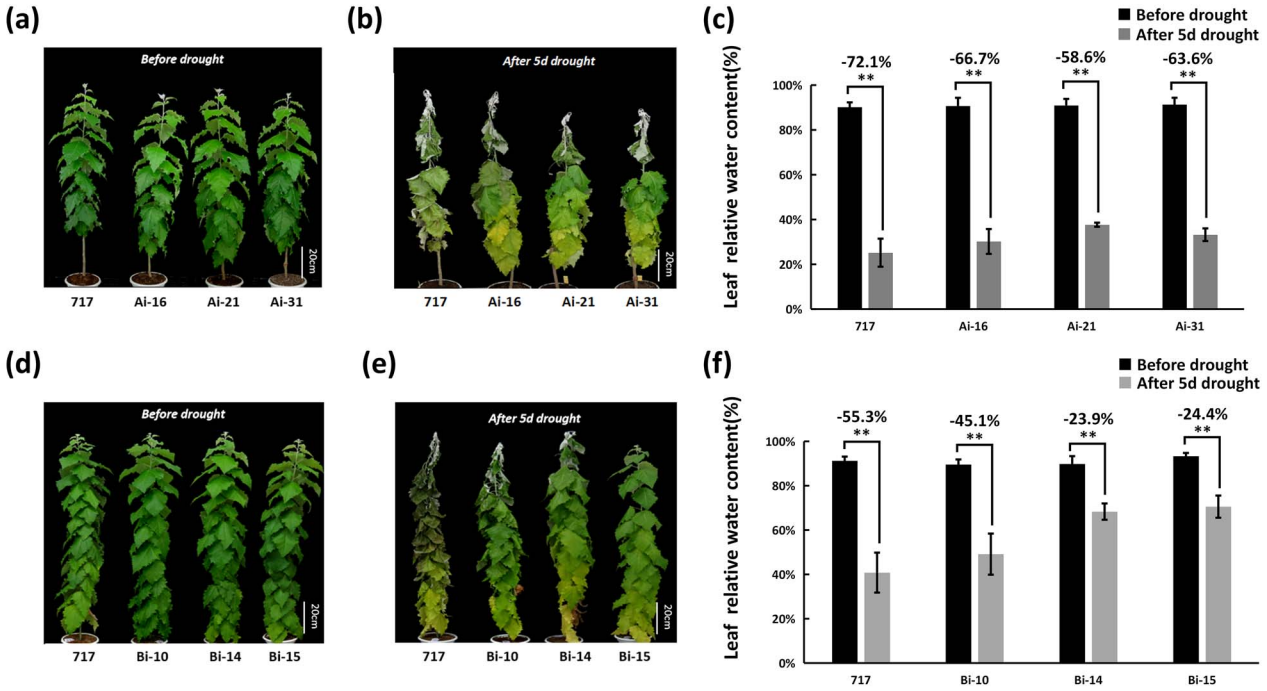

Figure 4. *PtKTI12A* or *PtKTI12B* RNAi plants were more tolerant to drought stress. (a)–(c) Plant morphology and leaf water content of *PtKTI12A* RNAi plants under drought stress. (d)–(f) Plant morphology and leaf water content of *PtKTI12B* RNAi plants under drought stress treatment. Values in poplar 717 control plants were set to 100% in (e and f); error bars represent SD, $n = 3$. ∗ and ∗∗significant at $P < 0.05$ and $P < 0.01$, respectively, by Student's t-test.

Taken together, our data support PtKTI12A and PtKTI12B proteins as an active partner of the Elongator complex regulating tRNA wobble uridine modification in hybrid poplar. More importantly, these genes are actively regulaged by drought and heat stress, and down-regulation of *PtKTI12A/12B* improve poplar tolerance against drought stress (Figure 8). What genes/proteins are affected by the defects on wobble uridine modifications (ncm^5U and others), and the mechanism concerning metabolites and gene expression regulation behind the drought phenotype, awaits further investigation.

Discussion

PtKti12A/12B proteins are involved in Populus response to abiotic stress

Interestingly, *PtKTI12A/12B* down-regulation led to better drought tolerance in transgenic plants. This phenotype is similar to the previous finding in *Arabidopsis*, where *elp1* mutant was more tolerant of drought stress (Chen Q et al. 2006). According to the changes of various physiological indicators during drought stress, we believe that down-regulation of *PtKTI12A or PtKTI12B*, transgenic plants showed higher drought stress tolerance (Figure 5). Indeed, qRT-PCR data showed that *PtKTI12A* and *PtKTI12B* could be up-regulated remarkably by both drought and heat stress, the transcript levels increasing sharply after 11 days after drought stress and then declineing back to normal after 13 days; similarly under heat stress an increased window of about 3 h was observed (Figure 2c–f). Although temporal transcriptional regulation upon various stresses is common in plants, we hypothesize that during these 'adaptation windows', that is, ca. 9–13 days after drought stress, and 1–4 h after heat stress, poplar seedlings undergo fast dynamic changes both on gene expression and tRNA nucleoside modifications. These cellular events lead to changes in protein translation and mediate the synthesis of necessary metabolites and signal molecules to help poplar to adapt to the new environment and establish stress resistance (Figure 8). Indeed, the extent of changes on gene expression during heat stress is more dramatic than drought stress; therefore, we would in the future be more interested to compare the heat tolerance between transgenic trees and control plants when they are in pots or in the field.

PtKti12A/12B proteins participate in tRNA wobble uridine modifications

ELP and *KTI* genes have been well studied in *S. cerevisiae* for their function in wobble uridine modification and killer toxin sensitivity, since the presence of ncm^5- or mcm^5-side chains and s^2 group on wobble uridine in specific tRNAs determines whether they can be cleaved by endo-nuclease zymocin (Huang et al. 2005, Huang et al. 2008, Lu et al. 2005, 2008). Our data suggest that PtKti12A/12B proteins also participate

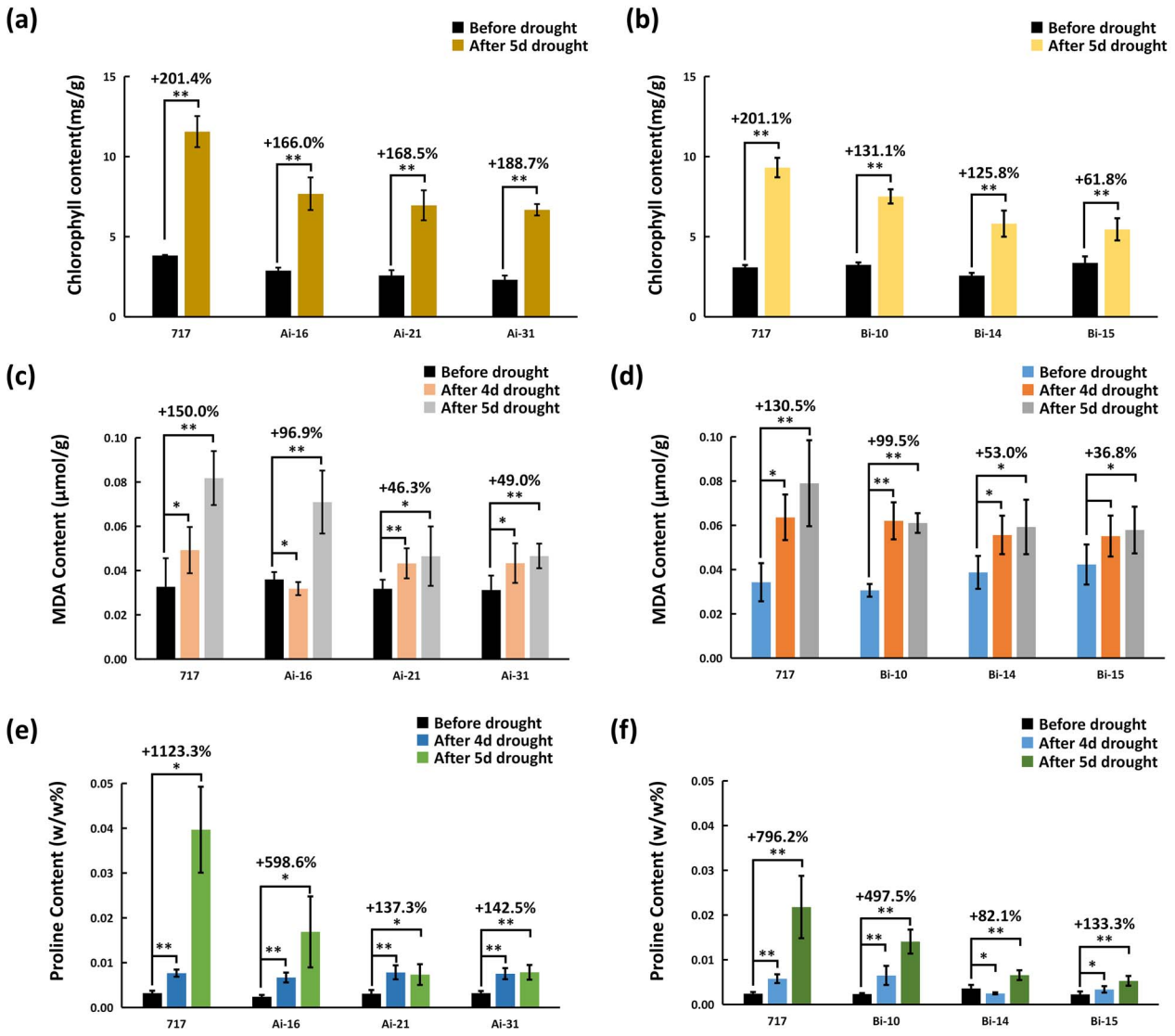

Figure 5. Chlorophyll, proline and MDA contents upon drought stress treatment. (a and b) Total chlorophyll contents before and 5 days after drought treatment in *PtKTI12A* and *PtKTI12B* RNAi lines. (c) and (d) Proline content (w/w %) before and 5 days after drought treatment in *PtKTI12A* and *PtKTI12B* RNAi lines. (e) and (f) MDA content before and 5 days after drought treatment in *PtKTI12A* and *PtKTI12B* RNAi lines. Error bars represent SD, $n = 3$. $*$ and $**$ significant at $P < 0.05$ and $P < 0.01$, respectively, by Student's t-test.

in tRNA wobble uridine modification as do their counterparts in *Arabidopsis* (Chen et al. 2010, Mehlgarten et al. 2010). Together with the sequence homology and structural model similarity between poplar Kti12 proteins with the other known Kti12 orthologs, we hypothesize that in woody plant *Populus*, the Kti12A/12B proteins form a functional holo-enzyme with the corresponding Elp1-6 proteins, and participates in the biosynthetic pathway of ncm^5U, mcm^5U and mcm^5s^2U modifications at wobble positions of target tRNA substrates.

Previous work showed that the function of subunits of the Elongator complex on wobble uridine modification is conserved between yeast and *Arabidopsis* (Mehlgarten et al. 2010). Due to the close phylogenic relationship between *Populus* and *Arabidopsis*, it is perhaps not difficult to envision similar Elongator protein complex setups in poplar as it is the case in *Arabidopsis*. However, the poplar genome goes through evolutional duplication, the two PtKti12 proteins identified in this study showed different expression profiles. Yet, the similarity on PtKti12A/12B subcellular localization and protein tertiary structure prediction indicate that their function on wobble uridine modification is conserved (Figures 6 and 7). How PtKti12A/12B proteins physically interact with poplar Elp proteins awaits future study.

Kti12 and Elp proteins in poplar and perspective roles in higher plants

Xu et al. (2012) reported that the Elongator complex interacts with proliferating cell nuclear antigen required for histone acetylation, and through this affects DNA replication and mitotic

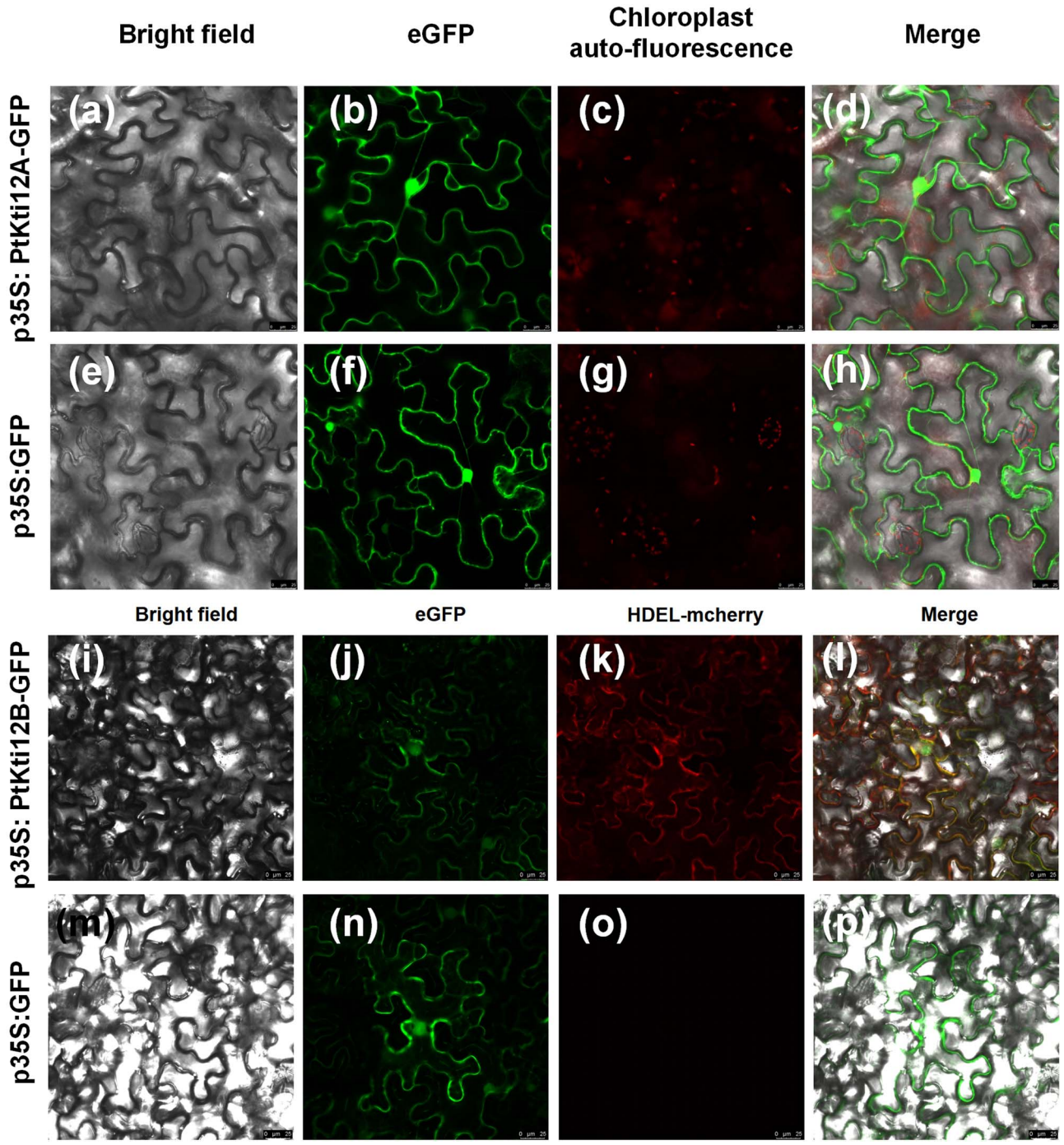

Figure 6. PtKti12A/12B proteins share similar subcellular localization. (a)–(h) PtKti12A subcellular localization. (a)–(d) PtKti12A-eGFP; (e)–(h) eGFP. From left to right: bright field, eGFP signal, chloroplast autofluorescence and merge of GFP and chloroplast autofluorescence signals. (i)–(p) PtKti12B subcellular localization. (i)–(h) PtKtil12B-eGFP; (m)–(p) eGFP. From left to right: bright field, eGFP signal, HDEL-mcherry (ER marker) and merge of GFP and mcherry signals. Scale bar = 25 μm.

cell cycle. Plant *elp* or *kti* mutants also showed phenotypes in meristem activity and organ growth, in leaf shape and polarity formation (Cho et al. 2007). Nelissen reported the *Arabidopsis* Elp3 protein regulates chromatin histone acetylation modification and transcription activity of auxin-related genes, hormone synthesis and transport within plant tissues were attributed to explain the vegetative growth phenotype observed in the corresponding mutants (Nelissen et al. 2010). Transcription factors, such as the class I KNOX genes, and genes involved in meristem growth and leaf polarity setup were also regulated by the Elongator complex, yet the nature of the regulatory mode is unclear (Kojima et al. 2011). During a comparison of mutants

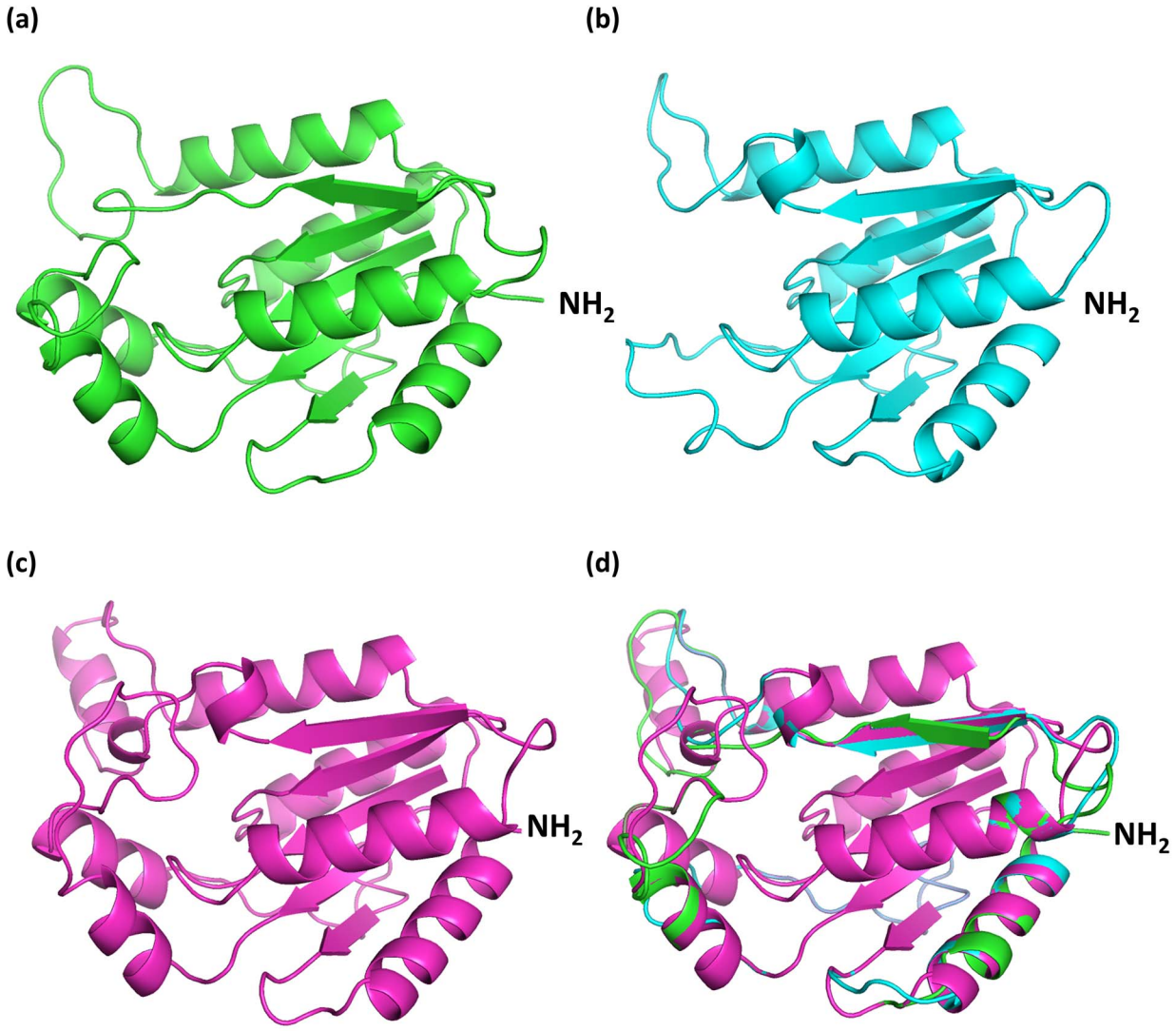

Figure 7. Protein tertiary structure prediction of PtKti12A, PtKti12B and CtKti12. PtKti12A protein N-terminal (a.a.2–178) structure. (a) PtKti12B protein N-terminal (a.a.2–158) structure (b) and CtKti12 protein N-terminal (a.a. 2–251) structure (c) were predicted by Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index). (d) Overlay of the three predicted structures: PtKti12A in green, PtKti12B in blue, CtKti12 in pink. Protein N-terminus is indicated by 'NH_2'.

defective in either the ncm_5-/mcm^5- or the s^2-side chain on wobble uridines, Nakai et al. (2019) showed that mutants devoid of the five-side chains suffered more than the ones lacking the s^2 group during drought stress conditions. However, both types of mutants had disordered leaf palisade mesophyll cells, and delayed endo-replication, possibly through the interaction of the same Elongator complex with proteins involved in DNA replication during leaf development (Nakai et al. 2019).

In *Arabidopsis* and tomato, much of the work has clearly shown that *ELP* or *KTI* genes can regulate organ growth, abiotic stress tolerance and immune response. However, no work has been reported for woody plants so far. We believe our work has shed some light on *Populus KTI12* genes on their function for tRNA wobble uridine modification and stress tolerance (Figure 8). However, many questions remain to be explored, such as their physical interactions with the Elongator complex subunits, and how these interactions regulate development and stress tolerance, possibly via changes in metabolites or proteins associated with tRNA wobble uridine modifications.

Conclusion

tRNA modification has been known for over a half-century; however, research on the function of tRNA modification in higher plants is scarce, especially in woody plants. In this study, we used a model woody plant *Populus* to identify and analyze the function of *KTI12* genes associated with the Elongator complex for tRNA wobble uridine modifications. In general, the two

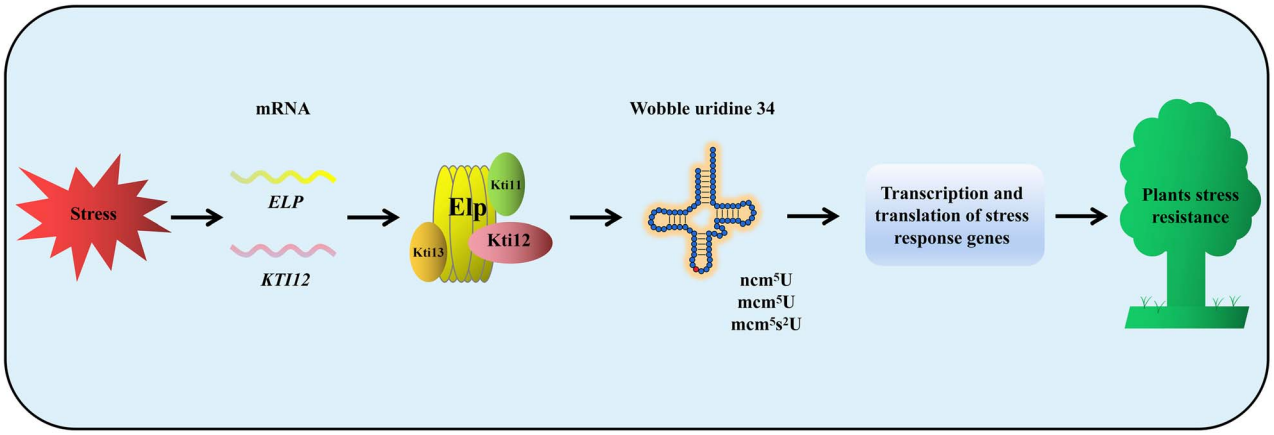

Figure 8. Hypothetical model for how Kti12 and Elp regulate plant stress tolerance. Environmental stresses induce transcript changes on *KTI12A/12B* gene, which leads to changes in the Elongator protein complex and function toward tRNA wobble uridine modification. Changes in protein translation subsequently influence metabolites and signal pathways leading to plant stress resistance.

KTI12 genes in *Populus* exhibited different expression profiles, but similar subcellular localization. Down-regulation of either *PtKTI12A* or *12B* gene resulted in the reduction of wobble uridine nucleosides. Plants with fewer *PtKTI12A/12B* transcripts showed no difference in growth rate under normal conditions and they displayed better drought tolerance. Our results support *PtKTI2A/12B* genes in association with tRNA wobble uridine modification and drought tolerance in hybrid poplar.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

Acknowledgment

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Author contributions

W.H. and X.C. performed the majority of the experimental work. Z.Y. and Y.X. were involved in the original construction and tissue-culture for generating various poplar transgene materials. J.X. and Y.X. helped with the analysis of nucleosides and gene expression by qRT-PCR. Z.B. gave advice for the project design and revised the manuscript. P.C. was responsible for all the data generated and manuscript writing. The manuscript was written by contributions from all authors. All authors have given approval to the final version of the manuscript.

Conflict of interest

None declared.

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