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Research article

Expression profiling of CTR1-like and EIN2-like genes in buds and leaves of *Populus tremula*, and *in vitro* study of the interaction between their polypeptides

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ABSTRACT

In Arabidopsis, the serine/threonine protein kinase Constitutive Triple Response 1 (CTR1) and Ethylene Insensitive 2 polypeptide (EIN2) functions are key negative and positive components, respectively, in the ethylene signalling route. Here, we report on an *in silico* study of members of the CTR1-like and EIN2-like polypeptide families from poplars. The expression of *CTR1*-like and *EIN2*-like genes such as *Ptre-CTR1*, *Ptre-CTR3* and *Ptre-EIN2a* was studied in *Populus tremula* buds and leaves in response to dehydration, various light conditions and under senescence. In buds under dehydration, the maximal fold-change of the *Ptre-CTR1*, *Ptre-CTR3* and *Ptre-EIN2a* expression level recorded almost identical values. This suggests that maintenance of a constant ratio between the transcript levels of genes encoding positive and negative ethylene signalling components is required under stress. The expression of the studied genes was 1.4-to 3-fold higher in response to darkness, but 4.5- to 51.2-fold and 21.6- to 51.2-fold higher under the early and moderate leaf senescence, respectively. It is worth noting that the senescence-related *Ptre-EIN2a* and *Ptre-CTR3a* expression profiles were very similar.

Using *in vitro* assays, we demonstrated the ability of the catalytic domain of Ptre-CTR1 to phosphorylate the Ptre-EIN2a-like polypeptide, which is similar to that in Arabidopsis. The target substrate, the Ptre-CEND2a polypeptide (C-terminal part of Ptre-EIN2a), was only phosphorylated by the protein kinase Ptre-CTR1 and not by Ptre-CTR3. Moreover, the addition of Ptre-CTR3 polypeptides (-CTR3a or -CTR3b forms) to the reaction mixture had an inhibitory effect on Ptre-CTR1 auto- and trans-phosphorylation. In contrast to Ptre-CTR1, Ptre-CTR3 may act as a positive regulator in ethylene signalling in poplar; however, this hypothesis requires *in vivo* confirmation. Thus, the ethylene signalling route in poplar seems to be under the control of certain additional mechanisms which have not been reported in Arabidopsis.

1. Introduction

In higher plants, ethylene acts as a critical mediator of plant growth, development and stress response. Its synthesis involves two steps: the first, from S-adenosylmethionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC) catalysed by 1-aminocyclopropane-1-carboxylate synthase (ACS); and the second, from ACC to ethylene catalysed by 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) (Abeles et al., 1992; Adams and Yang, 1979; Yang and Hoffman, 1984). Since the breakthrough works of the above-cited authors, remarkable progress in genetic and molecular analyses of the ethylene synthesis and signalling pathways has been made (for a review, see Booker and DeLong, 2015; Iqbal et al., 2017; Rodrigues et al., 2014; Vanderstraeten and Van Der Straeten, 2017).

In Arabidopsis, ethylene is perceived by a family of five ethylene receptors divided into subfamily 1 (ETR1 and ERS1) and subfamily 2 (EIN4, ETR2 and ERS2); and in other plants by their orthologues, whose number may differ in different species (Hua and Meyerowitz, 1998; for a review, see Gallie, 2015; Merchante et al., 2013). Upon binding with ethylene, the ethylene receptors become inactive, which in turn leads to

Abbreviations: ABA, abscisic acid; ACO, 1-Aminocyclopropane-1-Carboxylate Oxidase; ACS, 1-Aminocyclopropane-1-Carboxylate Synthase; CTR1, Constitutive Triple Response 1; EDR1, Enhanced Disease Resistance 1; EIN2, Ethylene Insensitive 2; ETR1, Ethylene Receptor 1; SAM, S-adenosyl-L-methionine

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the inhibition of CTR1 (Constitutive Triple Response 1, MAPKKK, a cytosolic serine/threonine protein kinase) and results in the subsequent activation of downstream positive regulators of ethylene responses (for a review, see Merchante et al., 2013). In Arabidopsis, upon the absence of ethylene, the active CTR1 directly phosphorylates EIN2 and inactivates its function as a key positive regulator in the ethylene signalling route (Ju et al., 2012; Qiao et al., 2012; Wen et al., 2012); the loss-of-function mutations in *EIN2* renders plants insensitive to ethylene (Alonso et al., 1999; Bisson and Groth, 2015; for a review, see Merchante et al., 2013; Zheng and Zhu, 2016). In the presence of ethylene, CTR1 becomes inactive, and the subsequent lack of EIN2 phosphorylation results in its proteolytic cleavage, and the movement of CEND2 (C-terminus of EIN2) into the nucleus, where it regulates the subsequent ethylene signalling components either directly or via other factors finally resulting in expression of the ethylene-responsive genes (Ju and Chang, 2015; Zhang et al., 2018, 2017 and 2016). Despite EIN2's basal function in the ethylene pathway, it is also considered a common node in other signalling pathways (Beaudoin et al., 2000; Ghassemian et al., 2000; Wang et al., 2007).

Three out of four tomato *CTR*-like genes (*LE-CTR1*, *LE-CTR3* and *LE-CTR4*) fully or partially complement *ctr1* mutation in Arabidopsis (Adams-Phillips et al., 2004; Leclercq et al., 2002; Zhong et al., 2008), while *LE-CTR2* seems to be more similar to *EDR1* (Enhanced Disease Resistance 1 serine/threonine protein kinase) than to the other *LE-CTRs*. As suggested by Yasumura et al. (2015) based on a study of CTR1-like polypeptide in *Physcomitrella patens*, the roles of CTR1-related proteins during land-plant evolution have been diversified, and the CTR1-related proteins from angiosperms have lost their ancestral ABA signalling function. In Arabidopsis, CTR1 seem to be active as a dimer; three to six autophosphorylation sites have been identified within its catalytic domain (Mayerhofer et al., 2012; 2011).

In contrast to ABA triggering senescence, ethylene is neither necessary nor sufficient for its occurrence, but acts as its modulator (Grbic and Bleecker, 1995; Jing et al., 2002; Lim et al., 2007; for a review, see Koyama, 2018, 2014; Schippers et al., 2015). Ethylene-promoted senescence may only occur in leaves that have reached a defined age; therefore, it depends on age-related changes (ARCs) to individual leaves (Jing et al., 2005; Schippers, 2015). The ctr1-1 loss-of-function mutant, which shows a continuous activation of the ethylene signalling route, exhibits a wild-type timing of leaf senescence; thus, it maintains its ARC-dependence (Jing et al., 2005). Initially, an effect of ethylene on senescence was demonstrated using ethylene-insensitive Arabidopsis mutants such as *ethylene resistant 1* (*etr1*), *ethylene insensitive 2* (*ein2*) and *ethylene insensitive 3* (*ein3*), and tomato with an antisense suppression of ACC oxidase; all of these showed a greater leaf longevity than the wild types (Wang et al., 2013; Grbic and Bleecker, 1995; John et al., 1995). Moreover, the *ctr1-1* and *ein2-5* mutants exhibited premature and delayed pistil senescence, respectively (Carbonell-Bejerano et al., 2011). In Arabidopsis, of the 826 SAGs (senescence-related genes) expressed in leaves, 75 appeared to be expressed in an EIN2-dependent manner, (Carbonell-Bejerano et al., 2011). Schippers et al. (2015) reported that EIN3 (a key transcription factor under the control of upstream located EIN2) affects expression of 269 SAGs, e.g. the chlorophyll catabolic genes (Qiu et al., 2015). It has been shown that, in the EIN2-EIN3-microRNA164-NAC2 signalling route, EIN3 directly represses expression of microRNA164 which results in an increased level of *NAC2* transcripts (NAC2, a positive senescence regulator, also called ORE1 or ANAC092), (Koyama, 2014; Li et al., 2013). Moreover, EIN2 appears to be active in the NO signalling route involved in dark-induced leaf senescence (Niu and Guo, 2012).

Recently, the involvement of some ethylene-related genes in early response to dehydration has been reported for soybean (Arraes et al., 2015), grapevine (Hopper et al., 2016), tomato (Egea et al., 2018) and Arabidopsis (Clauw et al., 2015). In conclusion, the expression of ethylene-related genes is up- or down-regulated depending on whether they participated in early or late response to water deficiency. In terrestrial plants, about 95% of water loss occurs through stomata (Schroeder et al., 2001). It seems that ethylene alone promotes stomata closure, whereas this closure is opposed by the concerted action of ethylene and other hormones (for a review, see Beguerisse-Diaz et al., 2012; Wilkinson and Davies, 2010, 2009). Its inhibitory role in guard cell control seems to be specific to the ABA response, while it does not affect dark-induced stomatal closure (for a review, see Acharya and Assmann, 2009).

This work is the first study in which the expression profiles of the genes encoding members of the CTR1 and EIN2 family have been characterised in response to dehydration, various light conditions and senescence in *Populus tremula* buds and leaves. Moreover, the protein kinase activity assay of Ptre-CTR1 polypeptide with Ptre-CEND2a as a substrate demonstrated that both proteins may act in poplar as the functional counterparts of the well-established ethylene signalling components CTR1 and EIN2 from Arabidopsis. Contrastingly, the role of the Ptre-CTR3 polypeptide seemed to be completely different from that of Ptre-CTR1, because the serine/threonine protein kinase activity of Ptre-CTR1 was inhibited by the presence of the Ptre-CTR3 polypeptide. Therefore, the functioning of Ptre-CTR3 as a positive ethylene signalling component may be considered; however, this needs further *in vivo* study. Nonetheless, ethylene signalling in poplar seems to be regulated by additional mechanisms which have not been found in Arabidopsis.

2. Materials and methods

2.1. Bioinformatical analysis

A comparative *in silico* study of CTR1-like proteins was performed on 9 CTR1-like polypeptide sequences from poplars and 29 ones from other dicots, whereas the EIN2-like proteins study– on 4 EIN2-like ones from poplar and 29 from other dicot and monocot species. The investigated CTR1-like and EIN2-like polypeptides belongs to those studied by various authors and those deposited in NCBI database as predicted CTR1-like or EIN2-like proteins, (Fig. 2 and Fig. 3, and Supplementary materials 1). The CTR1 and EIN2s trees were constructed from the complete protein sequence alignment of the above-mentioned polypeptides by the Neighbor-Joining method (Saitou and Nei, 1987) with bootstrapping analysis (500 replicates), (Felsenstein, 1985). The bootstrap values supporting the adjacent node are located beside the branches on the trees. The trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the JTT matrix-based method (Jones et al., 1992). Evolutionary analyses were conducted in MEGA 6 (Tamura et al., 2013). CTR1 and EIN2-like polypeptides deposited in NCBI database as predicted proteins were labelled by a Latin prefix of species and the last four letter of accession number, (details in Supplementary materials 1).

2.2. Plant material

2.2.1. Buds preparation

At the end of March, branch cuttings from wild-grown *P. tremula* trees located at the Adam Mickiewicz University campus Morasko (Poznań, Poland) were harvested and placed at room temperature (20–23 °C) in chambers filled with water. They were allowed to develop without water limitation for 8–12 days at natural daylight cycle. Selected apical breaking buds at similar stages of development were taken as single bud cuttings (intact buds with about 3 mm pieces of wood that were removed just prior to freezing of buds in liquid nitrogen) and placed in Petri dishes filled with medium A (medium A: 1 x MS with 2.5 mM MES pH 5.8). The 20 h lasting pre-treatment carried out at room temperature and natural daylight cycle started at 2.00 p.m. and finished at 10.00 a.m. on the next day.

After pre-treatment, the batches of buds (buds at the developmental

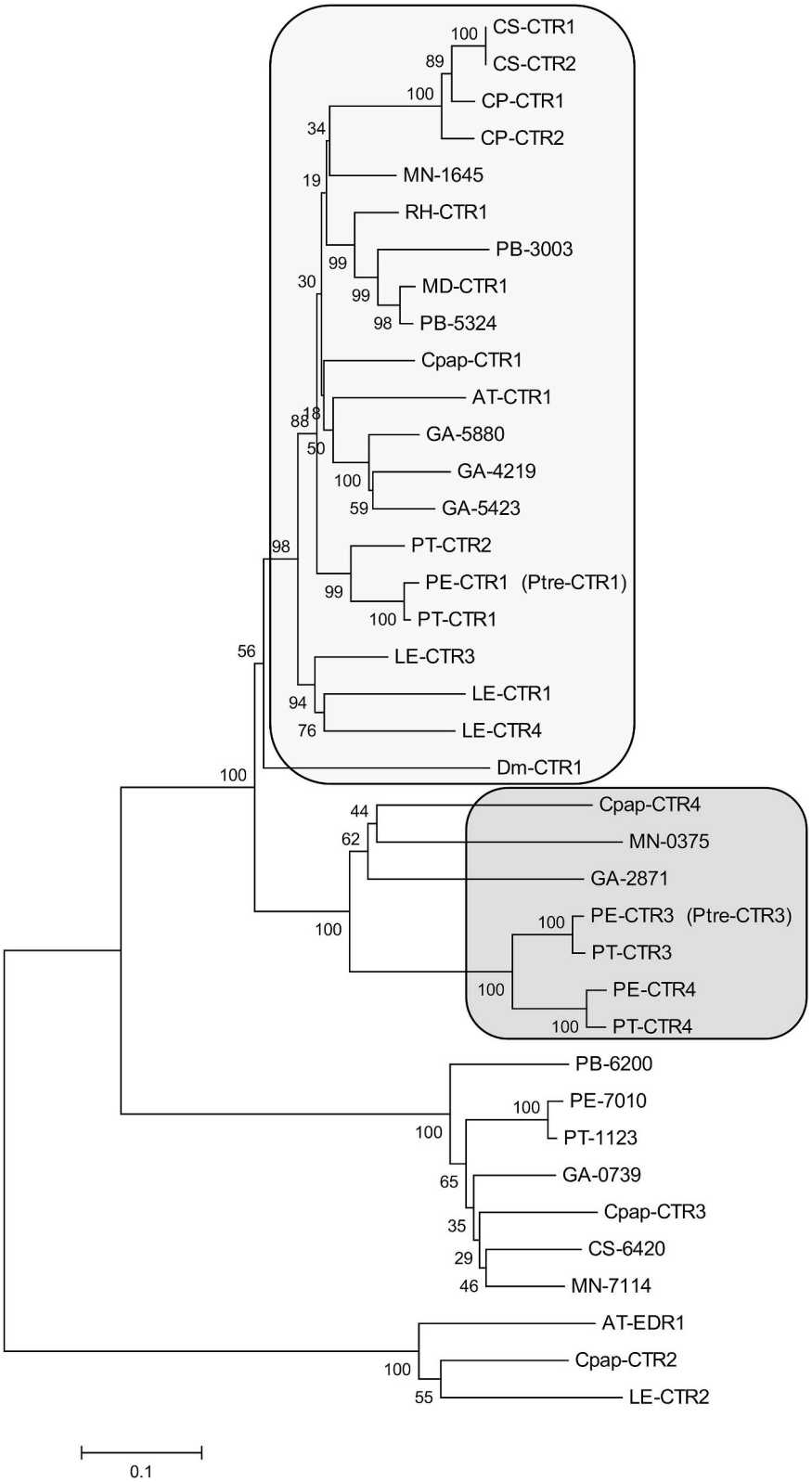

Fig. 1. A comparison of CTR1-like proteins from some dicot species: *Arabidopsis thaliana* (AT-); *Carica papaya* (Cpap-); *Cucurbita pepo* (CP-); *Cucumis sativus* (CS-); *Delphinium* (Dm-); *Gossypium arboreum* (GA-), *Lycopersicon esculentum* (LE-); *Malus domestica* (MD-); *Morus nobilis* (MN-); *Rosa hybrida* (RH-); *Populus euphratica* (PE-), *Populus tremula* (Ptre-), *Populus trichocarpa* (PT-) and *Pyrus* x *bretschneideri*, interspecies hybrid (PB-); the position of Ptre-CTR1 and Ptre-CTR3 polypeptides deduced from the high similarity of partial polypeptides to their counterparts from *P. euphratica*; details in Supplementary materials 1.

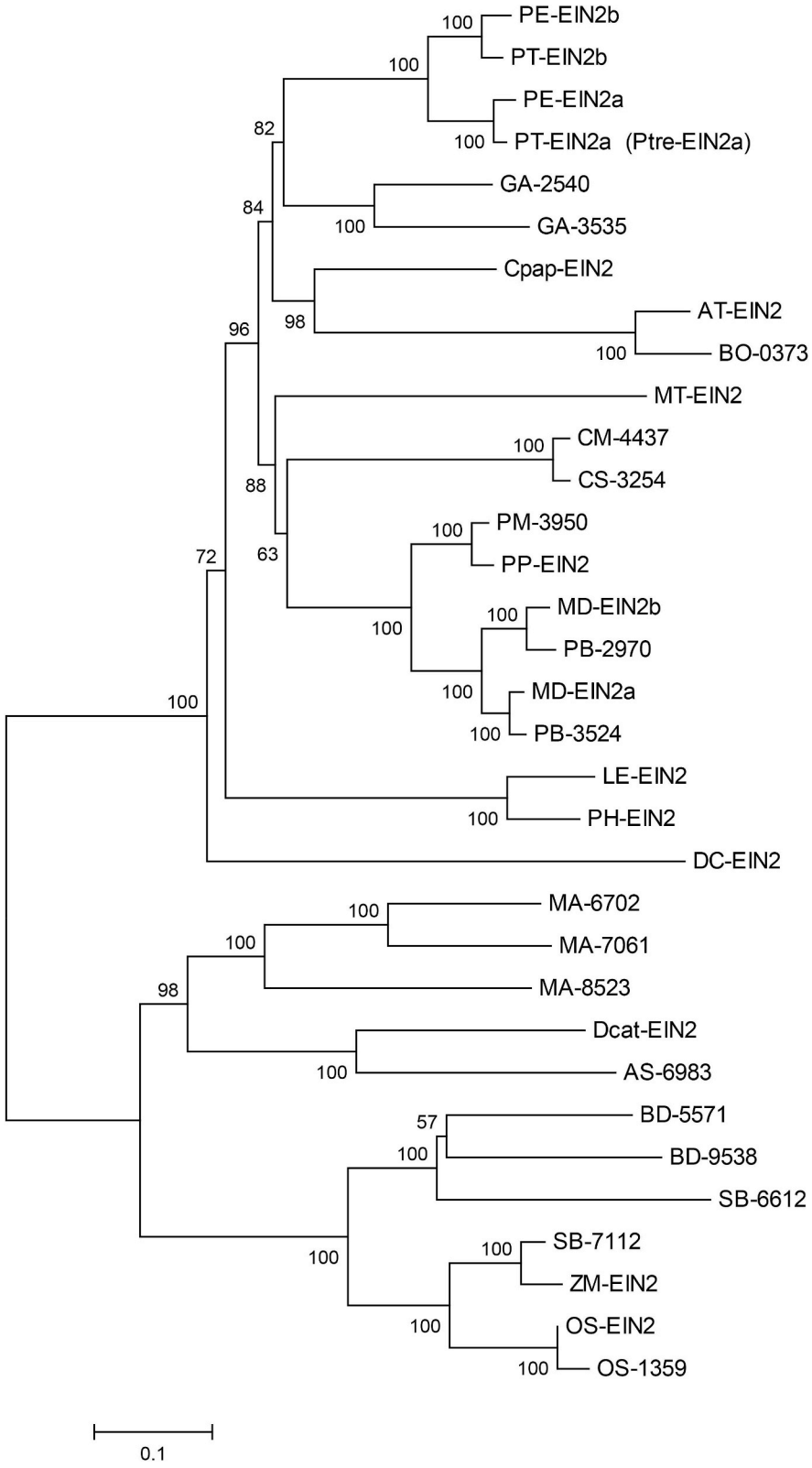

Fig. 2. A comparison of EIN2-like proteins from *Apostasia shenzhenica* (AS-), *Arabidopsis thaliana* (AT-); *Brachypodium distachyon* (BD-); *Brassica oleracea* (BO-); *Carica papaya* (Cpap-); *Cucumis sativus* (CS-); *Dendrobium catenatum* (Dcat-); *Dianthus caryophyllus* (DC-); *Gossypium arboreum* (GA-); *Lycopersicon esculentum* (LE-); *Malus domestica* (MD-); *Medicago truncatula* (MT-); *Musa acuminata* (MA-); *Oryza sativa* (OS-); *Petunia hybrida* (PH-); *Prunus mume* (PM-), *Prunus persica* (PP-), *Pyrus* x *bretschneideri* (PB-), *Populus euphratica* (PE-), *Populus tremula* (Ptre-), *Populus trichocarpa* (PT-), *Sorghum bicolor* (SB-) and *Zea mays* (ZM-); the position of Ptre-EIN2a polypeptide deduced from the high similarity of partial polypeptide to its counterpart from *P. trichocarpa*: details in Supplementary materials 1.

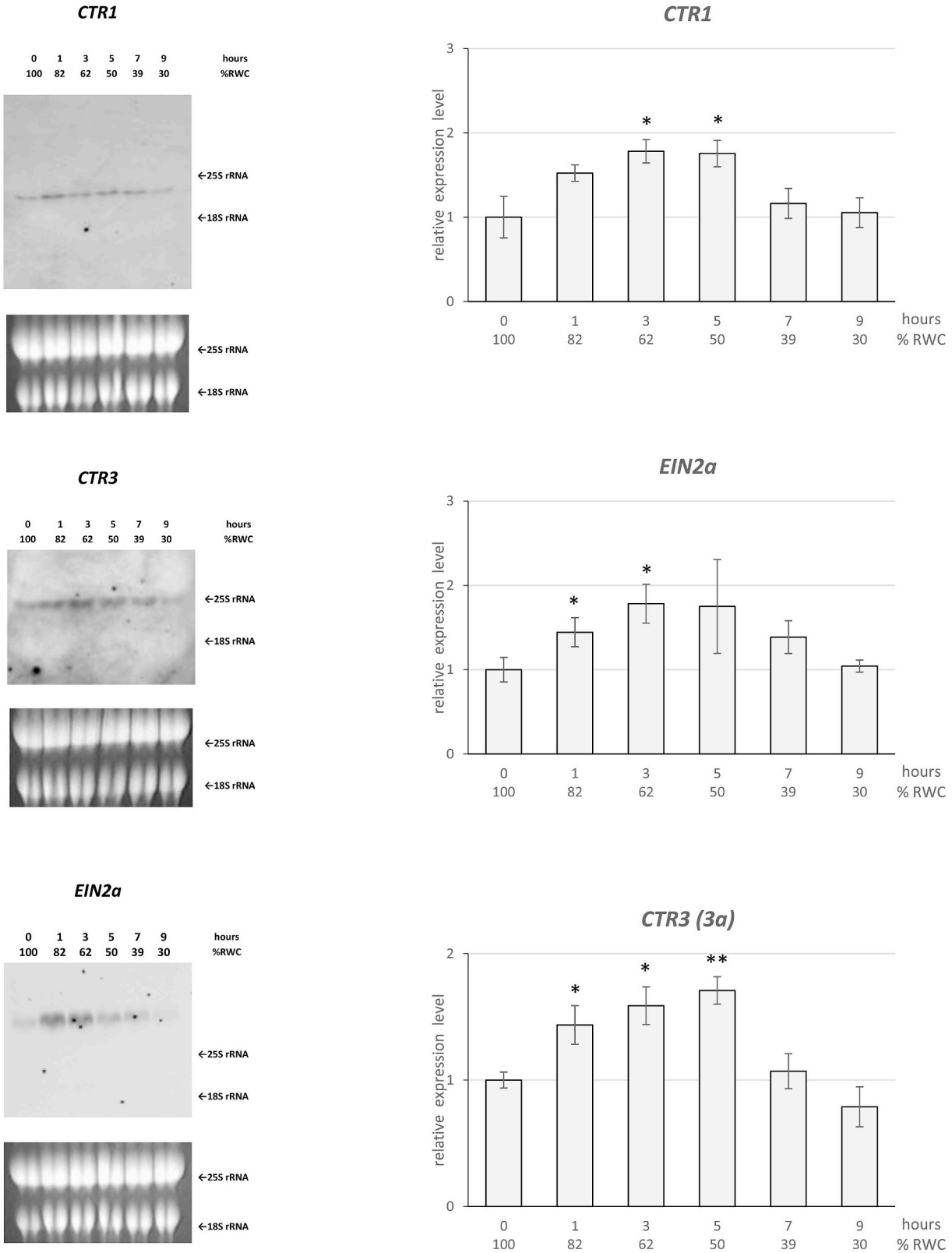

Fig. 3. The RNA blot analysis of the time course of *Ptre-CTR1, Ptre-CTR3* and *Ptre-EIN2a* gene expression in dehydrated buds using equal amounts of total RNA samples; for details see the materials and method section, and Supplementary materials 3.

(caption on next page)

Fig. 4. Relative quantitative expression profiling of the *P. tremula* ethylene signalling genes *Ptre-CTR1*, *Ptre-CTR3* (for *CTR3a* and *CTR3b* forms) and *Ptre-EIN2a* in dehydrated buds; columns represent the fold difference in gene expression in comparison to that in saturated buds (at 100% RWC), which was assessed as 1.0; the expression values for each sample were obtained from at least three biological replicates and error bars representing the standard deviation gene transcripts were normalized by elongation factor 1 reference gene expression; statistical analysis was performed using Student's t-test and the P_{values} were provided (*P < 0.05; **P < 0.01; ***P < 0.001); for details see the methods and Supplementary materials 2.

step of "breaking bud" were chosen, each batch contained 3 buds) were allowed to wilt while spread in flat cuvettes and kept at room temperature (21–23 °C) at relative humidity (RH) 55–58% at daylight. The dehydration experiments started at 10.00 a.m. and finished at 9.00 p.m. (mid April sunrise and sunset, 6.06 a.m. and 9.58 p.m., respectively) and buds were collected at 1 h intervals from 0 (control) to 9 h. After treatment the plant material was frozen in liquid nitrogen and stored at −80 °C.

In concomitant control experiment the relative water content (RWC) values were assessed. In our work, the RWC value was used as a measure of the degree of dehydration. The RWC was calculated as follows: RWC (%) = (fresh weight – dry weight) x 100/(saturated fresh weight – dry weight). The first measurement of fresh weight of batches of buds was performed immediately after cutting prior to their placement in medium A; the measurement of the saturated fresh weight was carried out after 20 h incubation in medium A. Under the course of dehydration of saturated buds, the gradual loss of fresh weight was measured. After dehydration course, the batches of buds were completely dried to assess the dry weight. In the breaking buds used in this work, the average value of RWC prior to their placement in medium A was 83.19 ± 3.74%; the average value of a dry mass content calculated versus their saturated state was 16.69 ± 0.59%.

2.2.2. Leaves preparation

Adult medium-size leaves without visible damages were collected in the mid of July. They were placed at vertical position in identical opened glass chambers (7 cm in diameter and 11 cm of high) in such a way that their petioles were soaked in 25 mL of medium A at the bottom and they were exposed to natural light conditions. The 24 h lasting treatments started at July 11th 5.00 p.m. and finished at 5.00 p.m. on the next day; July12th sunrise/sunset: 4.56 a.m./9.17 p.m.; temperature 25 °C ± 1; relative humidity about 55%. At the same time the identical treatment of leaves was carried out in the darkness. After the treatments the leaf blades were sampled (each sample contained the halves of three different leaf blades) and frozen in liquid nitrogen.

In the adult leaves used in this work, the average value of RWC (calculated as described for buds in section 2.2.1) prior to their placement in medium A was 86.86 ± 1.20%; whereas the average value of a dry mass content calculated against their saturated state (after 24 h lasting incubation in medium A) was 43.49 ± 0.84%.

2.2.3. Senescing leaves preparation

Medium-size leaves without visible damages from trees described in above sections were collected on 6 and 27 October between 2.30 and 4.00 p.m. (sunrise/sunset, 7.10 a.m., 8.29 p.m. and 7.46 a.m., 7.44 p.m., respectively) and grouped in such a manner that each sample contained two leave blades, and frozen with liquid nitrogen. The leaves collected on 6 October included leaves without any yellowing symptoms (representing the early senescence), while the leaves collected on 27 October exhibited partial yellowing, (details see Fig. S3). The average dry mass content was calculated versus saturated state as described earlier in 2.2.1.

2.3. RNA preparation

RNA preparation was performed as previously described (Jakubowicz et al., 2018).

2.4. RNA-blot analysis and probe preparation

RNA-blot analyses were realized using 20 µg of totRNA from breaking buds (totRNA extracted from 9 buds per line) isolated as described in Jakubowicz et al. (2018); the prestaining RNA procedure according to procedure reported by Ogretmenet al.,(1993) and Zhao et al. (2013); for RNA blotting procedure details see Jakubowicz et al. (2018). In this experiment, the cDNA clones for *Ptre-CTR1, Ptre-CTR3* and *Ptre-EIN2a* were used as probes which were prepared according to Babula et al. (2003), see Supplementary materials 3 for further details.

2.5. Reverse transcription

Reverse transcription was performed as previously described (Jakubowicz et al., 2018).

2.6. Gene specific primers, cDNA amplicons and the qPCR analysis

The list of primers used in cDNAs amplification and details of qPCR procedure are shown in Supplementary materials 2; the cDNAs of studied genes were confirmed by sequencing analysis and deposited in NCBI database as follows:*Ptre-CTR1* (MH356781), *Ptre-CTR3a* (MH356780) and *Ptre-EIN2a* (MH356782), moreover they are shown in Fig. S1.

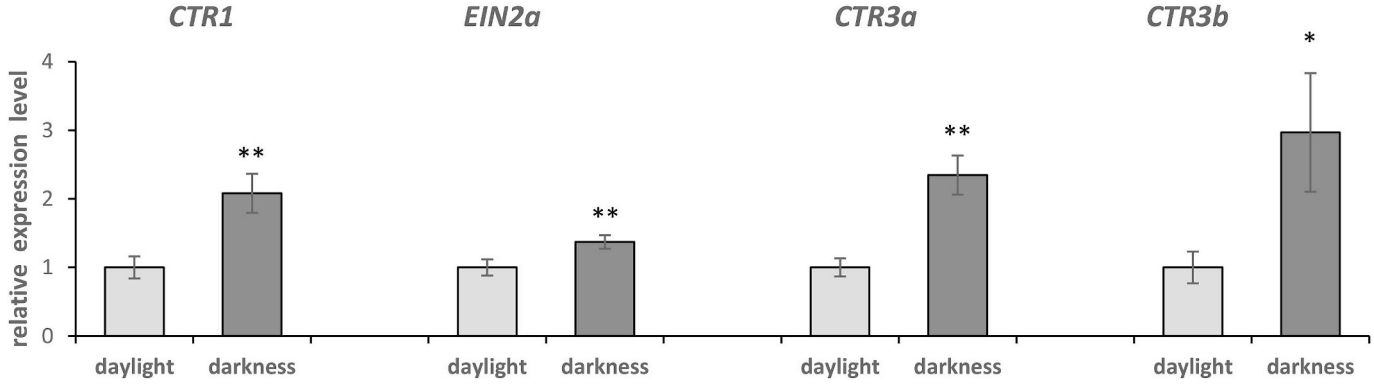

Fig. 5. Relative quantitative expression profiling of the *P. tremula* ethylene signalling genes *Ptre-CTR1, Ptre-EIN2a* and *Ptre-CTR3* (for *CTR3a* and *CTR3b* forms) in adult leaves exposed to daylight (light-shaded columns) or to darkness (dark-shaded columns); columns represent the fold difference in gene expression in comparison to that in daylight exposed leaves, which was assessed as 1.0. Other details as in Fig. 4 description.

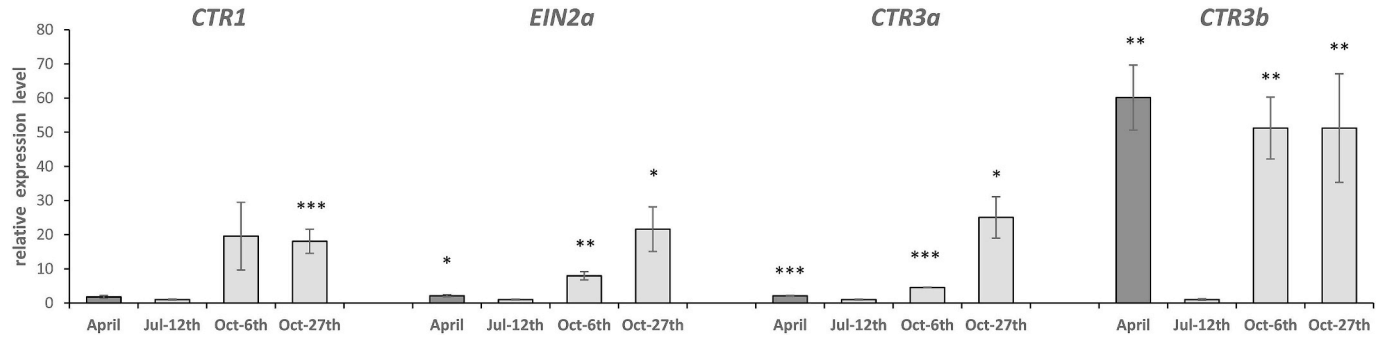

Fig. 6. Relative quantitative expression profiling of the *P. tremula* ethylene signalling genes *Ptre-CTR1, Ptre-EIN2a* and *Ptre-CTR3* (for *CTR3a* and *CTR3b* forms) in the saturated breaking buds (April), dark-shaded columns; the adult leaves (July-12th), the leaves at early phase of senescence (October-6th) and at moderate phase (October-27th), light shaded columns; columns represent the fold difference in gene expression in comparison to the adult leaves from July-12th, which was assessed as 1.0. Other details as in Fig. 4 description.

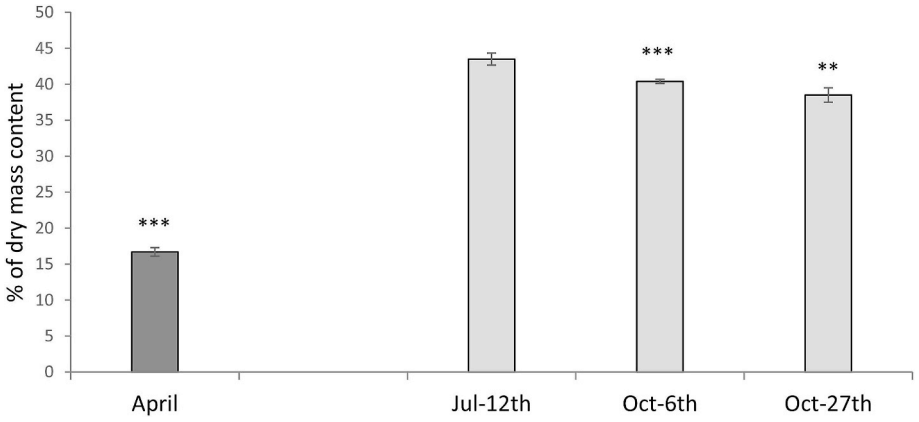

Fig. 7. The comparison of the dry mass content in the *P. tremula* breaking buds (April), dark-shaded column; in the adult leaves (July-12th), the leaves at early phase of senescence (October-6th) and at moderate phase (October-27th), light-shaded columns; columns represent the percentage content of dry mass; the statistical analysis (performed as described in Fig. 4) confirmed the differences between the dry mass content in the buds and adult leaves, in the adult leaves and those ones representing the early phase of senescence, and differences in the dry mass content at the early and moderate step of senescence.

2.7. qPCR data analysis

Quantification analysis was performed using StepOne software v2.3 (Applied Biosystems) with the standard curve method. In each assay for a specific cDNA target, standard curves were prepared using 6-fold serial dilutions of linear form of plasmid-cloned specific amplicon in 100 to 10^6 copies. Specific cDNA samples, standard cDNA samples and no-template controls were analyzed in three repeats in each assay. The specificity of products was validated by dissociation curve analyses. The relative expression levels of target genes were normalized to the expression of the constitutively expressed elongation factor 1 reference gene (Regier and Frey, 2010) and assessed as the ratio of target gene copies per 1000 copies of reference gene.

In poplar, the elongation factor 1 reference gene expression has been evidenced as stable in stem segments (Wang et al., 2015) and in roots under dehydration, (Regier and Frey, 2010), moreover reported as stable in roots and leaves in the other species (Galeano et al., 2014; Maroufi et al., 2010), thus it was independent on light conditions. In our study, the results of qPCR study on breaking buds with elongation factor 1 gene as a reference was in accordance with those from the RNA-blot analysis. The actin and tubulin gene expressions showed instability under studied conditions (not presented data).

2.8. Glutathione S-transferase (GST) or N-His-tag fused recombinant proteins

N-terminally GST- or His-tag fused fragments of target polypeptides are described below: kdPtre-CTR1 (1-267aa according to AMW11438) corresponds to catalytic domain (kd) in PE-CTR1 (550-817aa, XP_011018886) or to kdAT-CTR1 (557-821aa, NP_195993, At5g03730); Ptre-CTR3 (2-550aa according to AMW11437) corresponds to fragments of PE-CTR3 (127-675aa, XP_011032806) or AT-CTR1 (280-818aa); Ptre-CEND2a, C-terminal fragment of Ptre-EIN2a (1-595aa according to AMW11439) corresponds to PT-EIN2a (610-1204aa, XP_0063814444) or AT-EIN2 (608-1191aa, NP_195948, At5g03280).

In our work two forms of Ptre-CTR3 were denoted as Ptre-CTR3a and Ptre-CTR3b. Ptre-CTR3b polypeptide represents Ptre-CTR3 form lacking serine/threonine protein kinase catalytic domain (516-548aa or 659-691aa according to PE-CTR3 or AT-CTR1, respectively); Ptre-*CTR3a represents the C-terminal part of Ptre-CTR3a polypeptide lacking the ATP-binding site (431aa-675aa or 574-818aa according to PE-CTR3 or AT-CTR1, respectively). The details of construction, expression and purification of the used recombinant polypeptides in Supplementary materials 4 and Table S1.

2.9. In vitro protein kinase activity assays

Portions of 0.2–1 μg of GST- or His-tagged fragments of Ptre-CTR-like proteins were incubated (40 min, 25 °C) with 1 μg of Ptre-CEND2a polypeptide in a buffer containing 50 mM Tris(pH 7.5), 2 mM DTT, 10 mM $MgCl_2$, 1 mM adenosine- 5′- O- (3- thiotriphosphate) (ATP-γ-S, BIOLOG Life Science Institute, Bremen, Germany). After thiophosphorylation, 2.5mM*p*-Nitrobenzylmesylate (Abcam, Cambridge, UK) was added and samples were further incubated for 2 h at room temperature, separated by SDS-PAGE and subjected to immunoblotting (Allen et al., 2007) with anti-thiophosphate ester antibody (1:5000 dilution, Abcam, Cambridge, UK).

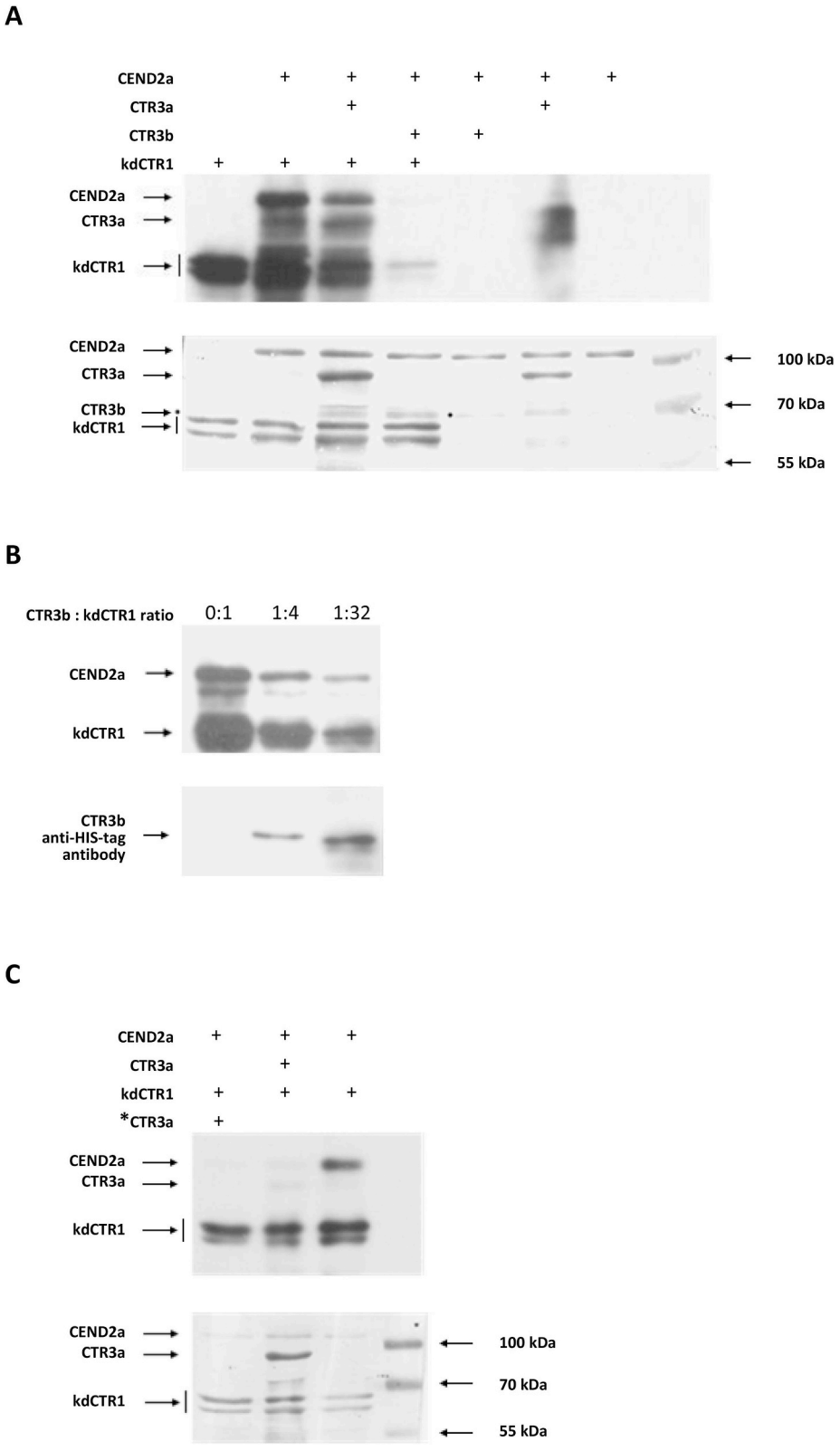

Fig. 8. *In vitro* kinase activity assay of Ptre-kdCTR1, Ptre-CTR3a, Ptre-*CTR3a and Ptre-CTR3b recombinants against of Ptre-CEND2a polypeptide.(A) from left to right: Ptre-kdCTR1 autophosphorylation; Ptre-CEND2a phosphorylation by Ptre-kdCTR1; the inhibitory effect of Ptre-CTR3a on the Ptre-CEND2a phosphorylation by Ptre-kdCTR1; the inhibitory effect of Ptre-CTR3b on autophosphorylation of Ptre-kdCTR1 and phosphorylation of Ptre-CEND2a by Ptre-kdCTR1; the lack of Ptre-CTR3b protein kinase activity; Ptre-CTR3a autophosphorylation and the lack of the phosphorylation of Ptre-CEND2a by Ptre-CTR3a; Ptre-CEND2a control; the protein size markers. At the bottom, protein loading control.(B) the inhibition of Ptre-kdCTR1 protein kinase activity by Ptre-CTR3b polypeptide in dose-dependent manner; from left to right: protein kinase activity of Ptre-kdCTR1 in the absence of Ptre-CTR3b; the decrease ofPtre-kdCTR1 protein kinase activity in the presence of the increasing amounts of Ptre-CTR3b polypeptide. At the bottom, the quantification of Ptre-CTR3b using anti-His-tag antibody.(C) from left to right: inhibition of Ptre-kdCTR1 protein kinase activity by Ptre-*CTR3a; inhibition ofPtre-kdCTR1 protein kinase activity by Ptre-CTR3a; Ptre-kdCTR1 protein kinase activity in the absence of Ptre-*CTR3a and Ptre-CTR3a. At the bottom, protein loading control; unfortunately, at used electrophoretic conditions the Ptre-*CTR3a protein (31.15 kDa) run off from 8% polyacrylamide gel.

3. Results

3.1. CTR1-like polypeptides

The functioning of the Constitutive Triple Response 1 (CTR1) serine/threonine protein kinase, an Raf-like MAPK kinase (MAPKKK) from Arabidopsis, as a negative regulator in ethylene signalling has been well-documented (see Introduction). The studied CTR-like polypeptides from dicot plants represent the putative counterparts of *A. thaliana* CTR1; nonetheless, their function has been poorly recognised at protein level. The only exception are four CTR1-like polypeptides from tomato (LE-CTR1-4), where LE-CTR2 has been verified as a potential EDR1-like protein (Adams-Phillips et al., 2004). The catalytic region of EDR1 (Enhanced Disease Resistance 1, serine/threonine protein Raf-like MAPKKK from plants, a relative of CTR1 functioning as a plant defence regulator) is similar to that from CTR1; therefore, the AT-EDR1 polypeptide was included in our *in silico* investigation. In the tree constructed for 38 polypeptides from dicot plants, 28 represent the main group divided into two subgroups involving 21 and 7 polypeptides. These 21 polypeptides (grey-shaded, Fig. 1) are the closest relatives of AT-CTR1 and they are denoted as the CTR1-like subgroup, whereas the other 7 polypeptides are denoted as CTR1-related (dark-shaded, Fig. 1); the next group, including 7 polypeptides, should be considered a non-CTR1-like group. Putatively, only some CTR1-like polypeptides reported by different authors and those deposited in NCBI database as predicted proteins (using the CTR1 specific signature of the ATP-binding site and typical Raf-like MAPKKKs motifs; for details, see Kieber et al., 1993) show a similar function to CTR1 from Arabidopsis. Unfortunately, some of the polypeptides reported as CTR1-like seem to be rather EDR1-like; for example, Cpap-CTR2 (Fig. 1).

The predicted amino acid sequences of Ptre-CTR1 and Ptre-CTR3 polypeptides were almost identical to their counterparts from *P. euphratica* (Fig. S1). Of the 11 polypeptides from poplars, 4 belong to the CTR1-like subgroup (PE-CTR1, Ptre-CTR1, PT-CTR1 and PT-CTR2), 5 to the CTR1-related subgroup (PE-CTR3, Ptre-CTR3, PE-CTR4, PT-CTR3 and PT-CTR4) and 2 seem to be non-CTR1-like (PE-7010 and PT-1123) (Fig. 1). The complete conservation of the counterparts of S/T residues functioning as self-directed activity targets in AT-CTR1 (S561, T564, S703, T704, S707 and S710) (Mayerhofer et al., 2012) was demonstrated in a CTR1-like subgroup from poplar (Fig. S2A). In those from the CTR1-related subgroup, S710 is substituted by N710 (according to PE-CTR3 N567) (S710 counterpart occurs only in PE-CTR4); moreover, the CTR3 ones do not conserve T714 localised at the beginning of subdomain VIII (714-721aa); they have R714 (according to PE-CTR3 R571) (Fig. S2A). In AT-CTR1, the interaction of D676 and K678 with T714 via hydrogen bonds is directly involved in substrate binding and recognition; furthermore, the primary role of S710 phosphorylation for AT-CTR1 function has been reported (Mayerhofer et al., 2012). In contrast, all the studied poplar polypeptides conserve aa residues, which the counterparts of the ones necessary for CTR1 dimerisation in Arabidopsis (Mayerhofer et al., 2012) (Fig. S2A). Thus, the possibility that they heterodimerise cannot be excluded. Moreover, the presence of the conserved G354 residue critical for CTR1-ETR1 interaction (Gao et al., 2003) has been confirmed in all studied poplar polypeptides (data not shown).

3.2. EIN2-like polypeptides

Our *in silico* study of 33 EIN2 polypeptides clearly illustrated a significant structural division between the EIN2 polypeptides from dicots and monocots, and demonstrated that they were encoded by one or two non-allelic gene copies, with as many as three genes in *M. acuminata*, (Fig. 2). In poplars, EIN2 is represented by two members, here denoted EIN2a and EIN2b; however, in this work, the presence of only Ptre-EIN2a mRNA was confirmed in buds and leaves. In Arabidopsis, CTR1 phosphorylates CEND2 (C-terminus of EIN2), and of particular interest are the robust phosphorylations of S645 and S924; hence, these phosphorylations take place only in the absence of ethylene (Chen et al., 2011; Ju et al., 2012; and Qiao et al., 2012). Less prominent phosphorylations have been demonstrated at S659, S739, S757, T819 and S1283 (Ju et al., 2012) and, according to Chen et al. (2011), at S743, S744, S748 and T742. The counterparts of the above-mentioned S/T residues are conserved in poplar EIN2a polypeptides, except for T742 and S757; whereas poplars are lacking the region including S1283. A comparison of the C-terminal parts of EIN2 from Arabidopsis and poplars is shown in Fig. S2B.

3.3. Expression profiles of transcripts encoding CTR1-and EIN2-like polypeptides in Populus tremula dehydrated buds

Of the identified ethylene signalling genes in poplar (see Figs. 1 and 2), the mRNAs of two CTR1-like genes (*Ptre-CTR1* and *Ptre-CTR3*) and one *Ptre-EIN2a* were detected in buds and leaves (details in Supplementary materials 2 and Fig. S1). Further study revealed that *Ptre-CTR3* mRNA is represented by two forms, which have been denoted by us as *CTR3a* and *-CTR3b (-CTR3b* is the shorter one, lacking exon 11). Of the prepared *Ptre-CTR3* cDNA clones (on the template totRNA from the same dehydrated buds as those used in the RNA-blot study, see Fig. 3), the twelve clones were sequenced; seven of them represented the *Ptre-CTR3a* form, while the others represented *Ptre-CTR3b* (see also Fig. S1B for further details). Our analysis of 20 genomic clones containing the fragment of *Ptre-CTR3* gene (from exon 6 to exon 13) did not reveal any examples of the lack of exon 11 or disturbance in adjacent regions (not shown). Moreover, an *in silico* study presented in the NCBI database for the *CTR3* transcript from *P. euphratica* (LOC105131500) predicts its seven isoforms, with one of them lacking exon 11 (XR_843450).

The study of expression patterns of *Ptre-CTR1, Ptre-CTR3* and *Ptre-EIN2a* genes through RNA-blot analysis of totRNA from dehydrated buds (for details, see Supplementary materials 3) showed that the size of the studied transcripts was in accordance with those predicted *in silico* for their counterparts from *P. euphratica* (*CTR1* and *CTR3*) and *P. trichocarpa* (*EIN2a*). Moreover, the results of the RNA-blot analysis demonstrated the stress induced a slight transient increase in mRNAs levels in the investigated genes (Fig. 3).

In the case of the *Ptre-CTR3* gene, a further qPCR study of its expression was carried out using the gene specific primers amplifying only *Ptre-CTR3a* or only *Ptre-CTR3b* cDNAs. The qPCR study revealed the enhancement of the *Ptre-CTR1*, *Ptre-CTR3a* and *Ptre-EIN2a* transcript levels beginning at the mild stress, and reaching the maxima at moderate dehydration; however, the *Ptre-CTR3b* level reached its peak at mild stress (Fig. 4).

3.4. Expression profiling of CTR1-and EIN2-like genes in adult Populus tremula leaves

The effect of exposition of the adult leaves (leaves collected in mid July, details in section 2.2.2) to daylight or to darkness on the expression of *CTR1*-and *EIN2*-like genes was studied.

The number of *Ptre-CTR1*, *Ptre-CTR3a, Ptre-EIN2a*, and *Ptre-CTR3b* transcripts was about 2.1-, 1.4-, 2.4- and 3-fold increased, respectively, in the dark versus the numbers in leaves exposed to daylight (159 ± 26; 94 ± 12; 28 ± 3; and 2.6 ± 0.6 copies at daylight, respectively) (Fig. 5). In daylight exposed leaves, the level of *Ptre-CTR3b* mRNA declined about 60-fold, while the levels of *Ptre-CTR3a* and *Ptre-EIN2a* mRNAs fell about 2.1-fold versus the levels in saturated buds (154 ± 24, 198 ± 13 and 59 ± 9 copies in buds, respectively) (Fig. 6).

3.5. Expression profiling of CTR1-and EIN2-like genes in Populus tremula senescing leaves

Under an age-dependent genetically programmed leaf senescence, the nutrients from leaves are translocated to storage tissues; thus, the dry mass of leaves gradually declines. Fig. 7 shows a comparison of dry mass content in the studied leaves. In our study, the leaves collected on 6 October did not exhibit any symptoms of yellowing (the early phase of senescence), while the leaves collected on 27 October were partially yellowed (the moderate step of senescence) (for details, see Fig. S3). The goal of our study was to compare the expression of *CTR1*-and *EIN2*-like genes at the both stages of leaf senescence. In leaves, at early/moderate stage of senescence, the abundance of the studied mRNAs, from the highest to the lowest number of transcripts, was as follows: *Ptre-CTR1*, 3121/2880; *Ptre-CTR3a*, 427/2357; *Ptre-EIN2a*, 223/606; and *Ptre-CTR3b*, 132/132, respectively.

At the early/moderate phase of senescence, the number of *Ptre-CTR1*, *Ptre-EIN2a*, *Ptre-CTR3a* and *Ptre-CTR3b* transcripts increased 19.6-/18.1-, 8.0-/21.6-, 4.5-/25.0-, and 51.2-/51.2-fold, respectively, versus the number in adult leaves from July (Fig. 6). Nonetheless, in the case of *Ptre-CTR1*, the observed increase in its level at the early phase was assessed as being statistically controversial (P_{value}0.115), because of the significant differences in transcript numbers in different biological replicates. The observed lability could indicate the early phase as being a critical time point for the *Ptre-CTR1* expression profile.

3.6. In vitro study of Ptre-CTR1, Ptre-CTR3 and Ptre-EIN2a interactions

An *in vitro* protein kinase assay was carried out to compare the properties of recombinant kdPtre-CTR1, Ptre-CTR3a, Ptre-*CTR3a and Ptre-CTR3b proteins. In the *in vitro* study of the recombinant proteins, phosphorylation of Ptre-CEND2a by kdPtre-CTR1 was observed. Ptre-CEND2a was not phosphorylated by Ptre-CTR3a; nonetheless, Ptre-CTR3a autophosphorylation was detected (Fig. 8). This means that, in our study, the recombinant kinases purified from *E. coli* entered the *in vitro* protein kinase assays in active form, which was in accordance with the observation of Mayerhofer et al. (2012) for recombinant CTR1 proteins from Arabidopsis but in contrast to the results of Huang et al. (2003). The lack of protein kinase activity for Ptre-CTR3b (Ptre-CTR3 form without catalytic core) and Ptre-*CTR3a (Ptre-CTR3a without ATP-binding site) was confirmed in the *in vitro* protein kinase assay (Fig. 8). Moreover, the addition of Ptre-CTR3a or Ptre-*CTR3a, or Ptre-CTR3b polypeptides to the reaction mixture of kdPtre-CTR1 and Ptre-CEND2a inhibited the kdPtre-CTR1 autophosphorylation and phosphorylation of Ptre-CEND2a by kdPtre-CTR1 (Fig. 8).

4. Discussion

In buds, independently of intracellular water content, the maintenance of a constant ratio between the *Ptre-CTR1* and *Ptre-EIN2a* mRNA levels seemed to be required, because the maximal change in their expression under dehydration versus that in saturated buds reached a identical fold-change value (i.e. 1.78) at the same time point in the course of dehydration (Fig. 4). The transcript levels of *Ptre-CTR3b* and *Ptre-CTR3a* reached their peaks after 1 h and 5 h of stress, respectively, with fold-change values of 1.42 and 1.71, respectively, (Fig. 4). Thus, the question as to whether both forms play similar or distinct functions in response to dehydration remains unanswered. A white-light dependent decrease in ethylene biosynthesis and white-light dependent decrease in ethylene-related gene expression have been reported by different authors (Vandenbussche et al., 2003; for a review, see Rodrigues et al., 2014). In our work, under continuous darkness lasting 24 hversus daylight conditions, the transcripts of studied genes exhibited 1.4–3.0-fold increases: with the lowest for *Ptre-EIN2a* and the highest for *Ptre-CTR3b* (Fig. 5). The essential increase in the *Ptre-CTR3b* mRNA level in buds and senescing leaves (Fig. 6) may suggest some function of Ptre-CTR3b polypeptide under these developmental steps. Moreover, the similarity of the senescence-related expression profiles of *Ptre-CTR3a* and *Ptre-EIN2a* implies that the Ptre-CTR3a polypeptide may participate in ethylene signalling.

Of the 48 Arabidopsis and 40 tomato members of the RAF-like subfamily of plant MAPKKKs (Jing et al., 2017), only one in Arabidopsis (CTR1, Constitutive Triple Response 1), and three in tomato (CTR1, 3 and 4) function as key negative regulators in the ethylene signalling pathway (Adams-Phillips et al., 2004; Kieber et al., 1993). In this work, of the 65 members of the RAF-like subfamily in poplar (Jing et al., 2017), we chose 5 different CTR1-like proteins for *in silico* study (Fig. 1); however, of the investigated -CTR1-4, only the *Ptre-CTR1* and *Ptre-CTR3* transcripts were detected in buds and leaves. Our *in vitro* assay of the S/T protein kinase activity of the kdPtre-CTR1 and Ptre-CTR3 polypeptides with Ptre-CEND2a polypeptide as the target substrate was performed in a similar manner as the studies reported for CTR1 and CEND2 from Arabidopsis (Ju et al., 2012; Qiao et al., 2012). The Ptre-CEND2a polypeptide conserves the counterparts of 8 of 11 S/T residues from AT-CEND2a, which have been evidenced to be phosphorylation targets for AT-CTR1 (Fig. S2B). The *in vitro* study performed in this work revealed that Ptre-CTR1 recognised and phosphorylated Ptre-CEND2a (Fig. 8), in contrast to the lack of Ptre-EIN2a phosphorylation by Ptre-CTR3a. The addition of Ptre-CTR3b or Ptre-CTR3a polypeptides, or Ptre-*CTR3a, to the reaction mixture had an inhibitory effect on kdPtre-CTR1 autophosphorylation and phosphorylation of Ptre-CEND2a by kdPtre-CTR1 (Fig. 8). Therefore, in Ptre-CTR3 polypeptides, the aa residues responsible for this inhibition should be localised in the 431-675aa region, with the exception of 516–548aa (for details, see section 2.8). All the residues located in the catalytic domain in AT-CTR1 reported as critical for S/T protein kinase function (Mayerhofer et al., 2012) are conserved in poplar polypeptides from the CTR1-like subgroup (the one including CTR1 from poplar and CTR1 from Arabidopsis) (Fig. 1 and Fig. S2A). In contrast, poplar polypeptides from the CTR1-related subgroup (including CTR3 and CTR4 from poplar) do not conserve some of these residues, such as S710, which was substituted by N710 (only PE-CTR4 had S710). Moreover, in CTR3s from poplars, the position corresponding to T714 is occupied by R714, (Fig. 1 and Fig. S2A). In AT-CTR1, T714 is directly involved in recognition and substrate binding (Mayerhofer et al., 2012). In Arabidopsis, CTR1 has been shown to function as a dimer (Mayerhofer et al., 2012). Both subgroups of poplar polypeptides (CTR1-like and CTR1-related) conserve the counterparts of the residues recognised in AT-CTR1 as essential for protein dimerisation. Thus, the members representing different subgroups could heterodimerise. From this point of view, in poplars, the Ptre-CTR3 polypeptides (-CTR3a and –CTR3b) could regulate Ptre-CTR1 activity or even play the role of positive regulators in the ethylene signalling pathway. Nonetheless, this requires further *in vivo* investigation.

In our work the expression profiles of the two key components of the ethylene signalling route were investigated in response to dehydration, various light conditions, and under senescence in *P. tremula* buds and leaves. Ptre-CTR1 and Ptre-EIN2a polypeptides were verified as the possible functional counterparts of the well-established ethylene signalling components CTR1 and EIN2 from Arabidopsis. Moreover, the role of the Ptre-CTR3 polypeptide as the positive ethylene signalling component was discussed. In conclusion, the ethylene signalling route in poplar seems to be under the control of certain additional mechanisms which have not been found in Arabidopsis.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.04.029.

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Disclosure

The authors have no conflicts of interest to declare.

Małgorzata Jakubowicz: conceptualization, methodology, investigation, writing – original draft, writing – review & editing, visualization, supervision.

Witold Nowak: validation, formal analysis, investigation, writing – review & editing, visualization.

Łukasz Gałgański: investigation, writing – original draft, visualization.

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References

Abeles, F.B.M., Morgan, P.W., Saltveit, M.E., 1992. Ethylene in Plant Biology. Academic Press, San Diego, CA.

Acharya, B.R., Assmann, S.M., 2009. Hormone interactions in stomatal function. Plant Mol. Biol. 69, 451–462. https://doi.org/10.1007/s11103-008-9427-0.

Adams, D.O., Yang, S.F., 1979. Ethylene biosynthesis – identification of 1-aminocyclopropane-1-carboxylic acid as an intermediate in the conversion of methionine to ethylene. Proc. Natl. Acad. Sci. U.S.A. 76, 170–174.

Adams-Phillips, L., Barry, C., Kannan, P., Leclercq, J., Bouzayen, M., Giovannoni, J., 2004. Evidence that CTR1-mediated ethylene signal transduction in tomato is encoded by a multigene family whose members display distinct regulatory features. Plant Mol. Biol. 54, 387–404.

Allen, J.J., Li, M., Brinkworth, C.S., Paulson, J.L., Wang, D., Hübner, A., Chou, W.H., Davis, R.J., Burlingame, A.L., Messing, R.O., Katayama, C.D., Hedrick, S.M., Shokat, K.M., 2007. A semisynthetic epitope for kinase substrates. Nat. Methods 4, 511–516.

Alonso, J.M., Hirayama, T., Roman, G., Nourizadeh, S., Ecker, J.R., 1999. EIN2, a bifunctional transducer of ethylene and stress responses in Arabidopsis. Science 284, 2148–2152.

Arraes, F.B.M., Beneventi, M.A., de Sa, M.E.L., Paixao, J.F.R., Albuquerque, E.V.S., Rockenbach Marin, S.R., Purgatto, E., Nepomuceno, A.L., Grossi-de-Sa, M.F., 2015. Implications of ethylene biosynthesis and signaling in soybean drought stress tolerance. BMC Plant Biol. 15, 213. https://doi.org/10.1186/s12870-015-0597-z.

Babula, D., Kaczmarek, M., Barakat, A., Delseny, M., Quiros, C.F., Sadowski, J., 2003. Chromosomal mapping of *Brassica oleracea* based on ESTs from *Arabidopsis thaliana*: complexity of the comparative map. Mol. Genet. Genom. 268, 656–665.

Beaudoin, N., Serizet, C., Gosti, F., Giraudat, J., 2000. Interactions between abscisic acid and ethylene signaling cascades. Plant Cell 12, 1103–1115.

Beguerisse-Diaz, M., Hernandez-Gomez, M.C., Lizzul, A.M., Barahona, M., Desikan, R., 2012. Compound stress response in stomatal closure: a mathematical model of ABA and ethylene interaction in guard cells. BMC Syst. Biol. 6, 146.

Bisson, M.M.A., Groth, G., 2015. Targeting plant ethylene responses by controlling essential protein–protein interactions in the ethylene pathway. Mol. Plant 8, 1165–1174.

Booker, M.A., DeLong, A., 2015. Producing the ethylene signal: regulation and diversification of ethylene biosynthetic enzymes. Plant Physiol. 169, 42–50.

Carbonell-Bejerano, P., Urbez, C., Granell, A., Carbonell, J., Perez-Amador, M.A., 2011. Ethylene is involved in pistil fate by modulating the onset of ovule senescence and the GA-mediated fruit set in Arabidopsis. BMC Plant Biol. 11, 84. www.biomedcentral.com/1471-2229/11/84.

Chen, R., Binder, B.M., Garrett, W.M., Tucker, M.L., Chang, C., Cooper, B., 2011. Proteomic responses in *Arabidopsis thaliana* seedlings treated with ethylene. Mol. Biosyst. 7, 2637–2650.

Clauw, P., Coppens, F., De Beuf, K., Dhondt, S., Van Daele, T., Maleux, K., Storme, V., Clement, L., Gonzalez, N., Inze, D., 2015. Leaf responses to mild drought stress in natural variants of Arabidopsis. Plant Physiol. 167, 800–816.

Egea, I., Albaladejo, I., Meco, V., Morales, B., Sevilla, A., Bolarin, M.C., Flores, F.B., 2018. The drought-tolerant *Solanum pennellii* regulates leaf water loss and induces genes involved in amino acid and ethylene/jasmonate metabolism under dehydration. Sci. Rep. 8, 2791. https://doi.org/10.1038/s41598-018-21187-2.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.

Galeano, E., Vasconcelos, T.S., Ramiro, D.A., De Martin, V.F., Carrer, H., 2014. Identification and validation of quantitative real-time reverse transcription PCR reference genes for gene expression analysis in teak(*Tectona grandis*L.f.). BMC Res. Notes 7, 464. www.biomedcentral.com/1756-0500/7/464.

Gallie, D.R., 2015. Ethylene receptors in plants - why so much complexity? Prime Rep. 7, 39. https://doi.org/10.12703/P7-39.

Gao, Z., Chen, Y.F., Randlett, M.D., Zhao, X.C., Findell, J.l., Kieber, J., Schaller, G.E., 2003. Localization of the Raf-like kinase CTR1 to the endoplasmic reticulum of Arabidopsis through participation in ethylene receptor signaling complexes. J. Biol. Chem. 278, 34725–34732.

Ghassemian, M., Nambara, E., Cutler, S., Kawaide, H., Kamiya, Y., McCourt, P., 2000. Regulation of abscisic acid signaling by the ethylene response pathway in Arabidopsis. Plant Cell 12, 1117–1126.

Grbic, V., Bleecker, A.B., 1995. Ethylene regulates the timing of leaf senescence in Arabidopsis. Plant J. 8, 595–602.

Hopper, D.W., Ghan, R., Schlauch, K.A., Cramer, G.R., 2016. Transcriptomic network analyses of leaf dehydration responses identify highly connected ABA and ethylene signaling hubs in three grapevine species differing in drought tolerance. BMC Plant Biol. 16, 118. https://doi.org/10.1186/s12870-016-0804-6.

Hua, J., Meyerowitz, E.M., 1998. Ethylene responses are negatively regulated by a receptor gene family in *Arabidopsis thaliana*. Cell 94, 261–271.

Huang, Y., Li, H., Hutchison, C.E., Laskey, J., Kieber, J.J., 2003. Biochemical and functional analysis of CTR1, a protein kinase that negatively regulates ethylene signaling in Arabidopsis. Plant J. 33, 221–233.

Iqbal, N., Khan, N.A., Ferrante, A., Trivellini, A., Francini, A., Khan, M.I.R., 2017. Ethylene role in plant growth, development and senescence: interaction with other phytohormones. Front. Plant Sci. 8, 475. https://doi.org/10.3389/fpls.2017.00475.

Jakubowicz, M., Nowak, W., Gałgański, Ł., Babula-Skowrońska, D., 2018. Expression profiling of genes encoding ABA route components in response to dehydration or various light conditions in poplar buds and leaves. J. Plant Physiol. 223, 84–95.

Jing, H., Sturre, M.J.G., Hille, J., Dijkwel, P.P., 2002. Arabidopsis onset of leaf death mutants identify a regulatory pathway controlling leaf senescence. Plant J. 32, 51–63.

Jing, H.C., Schippers, J.H., Hille, J., Dijkwel, P.P., 2005. Ethylene-induced leaf senescence depends on age-related changes and old genes in Arabidopsis. J. Exp. Bot. 56, 2915–2923.

Jing, T., Wang, L., Liu, H., Wuyun, T., Du, H., 2017. Genome-wide identification of Mitogen-activated Protein Kinase cascade genes and transcriptional profiling analysis during organ development in *Eucommia ulmoides*. Sci. Rep. 7, 17732. https://doi.org/10.1038/s41598-017-17615-4.

John, I., Drake, R., Farrell, A., Cooper, W., Lee, P., Horton, P., et al., 1995. Delayed leaf senescence in ethylene-deficient ACC-oxidase antisense tomato plants: molecular and physiological analysis. Plant J. 7, 483–490.

Jones, D.T., Taylor, W.R., Thornton, J.M., 1992. The rapid generation of mutation data matrices from protein sequences. Comput. Appl. Biosci. 8, 275–282.

Ju, C., Chang, C., 2015. Mechanistic insights in ethylene perception and signal transduction. Plant Physiol. 169, 85–95.

Ju, C., Yoon, G.M., Shemanskya, J.M., Lina, D.Y., Ying, Z.I., Chang, J., Garrett, W.M., Kessenbrock, M., Groth, G., Tucker, M.L., Cooper, B., Kieber, J.J., Chang, C., 2012. CTR1 phosphorylates the central regulator EIN2 to control ethylene hormone signaling from the ER membrane to the nucleus in Arabidopsis. Proc. Natl. Acad. Sci. U. S. A 109, 19486–19491.

Kieber, J.J., Rothenberg, M., Roman, G., Feldmann, K.A., Ecker, J.R., 1993. CTR1, a negative regulator of the ethylene response pathway in Arabidopsis, encodes a member of Raf family of protein kinase. Cell 72, 427–551.

Koyama, T., 2014. The roles of ethylene and transcription factors in the regulation of onset of leaf senescence. Front. Plant Sci. https://doi.org/10.3389/fpls.2014.00650. article 650.

Koyama, T., 2018. A hidden link between leaf development and senescence. Plant Sci. 276, 105–110.

Leclercq, J., Adams-Phillips, L.C., Zegzouti, H., Jones, B., Latche, A., Giovannoni, J.J., Pech, J., Bouzayen, M., 2002. LeCTR1, a tomato CTR1-like gene, demonstrates ethylene signaling ability in Arabidopsis and novel expression patterns in tomato. Plant Physiol. 130, 1132–1142.

Li, Z., Peng, J., Wen, X., Guo, H., 2013. Ethylene-insensitive 3 is a senescence-associated gene that accelerates age-dependent leaf senescence by directly repressing miR164 transcription in Arabidopsis. Plant Cell 25, 3311–3328.

Lim, P.O., Kim, H.J., Nam, H.G., 2007. Leaf senescence. Annu. Rev. Plant Biol. 58, 115–136.

Maroufi, A., Van Bockstaele, E., De Loose, M., 2010. Validation of reference genes for gene expression analysis in chicory (*Cichorium intybus*) using quantitative real-time PCR. BMC Mol. Biol. 15. www.biomedcentral.com/1471-2199/11/15.

Mayerhofer, H., Mueller-Dieckmann, C., Mueller-Dieckmann, J., 2011. Cloning, expression, purification and preliminary X-ray analysis of the protein kinase domain of constitutive triple response 1 (CTR1) from *Arabidopsis thaliana*. Acta Crystallogr. Sect. F Struct. Biol. Commun. 67, 117–120.

Mayerhofer, H., Panneerselvam, S., Mueller-Dieckmann, J., 2012. Protein kinase domain of CTR1 from *Arabidopsis thaliana* promotes ethylene receptor cross talk. J. Mol. Biol. 415, 768–779.

Merchante, C., Alonso, J.M., Stepanova, A.N., 2013. Ethylene signaling: simple ligand, complex regulation. Curr. Opin. Plant Biol. 16, 554–560.

Niu, Y.H., Guo, F.Q., 2012. Nitric oxide regulates dark-induced leaf senescence through EIN2 in Arabidopsis. J. Integr. Plant Biol. 54, 516–525.

Ogretmen, B., Ratajczak, H., Kats, A., Stark, B.C., Gendel, S.M., 1993. Effects of staining of RNA with ethidium bromide before electrophoresis on performance of northern blots. Biotechniques 14, 932–935.

Qiao, H., Shen, Z., Huang, S.S., Schmitz, R.J., Urich, M.A., Briggs, S.P., et al., 2012. Processing and subcellular trafficking of ER-tethered EIN2 control response to

ethylene gas. Science 338, 390–393.
Qiu, K., Li, Z., Yang, Z., Chen, J., Wu, S., Zhu, X., Gao, S., Gao, J., Ren, G., Kuai, B., et al., 2015. EIN3 and ORE1 accelerate degreening during ethylene-mediated leaf senescence by directly activating chlorophyll catabolic genes in Arabidopsis. PLoS Genet. 11, e1005399.
Regier, N., Frey, B., 2010. Experimental comparison of relative RT-qPCR quantification approaches for gene expression studies in poplar. BMC Mol. Biol. 11, 57.
Rodrigues, M.A., Bianchetti, R.E., Freschi, L., 2014. Shedding light on ethylene metabolism in higher plants. Front. Plant Sci. 5, 665. https://doi.org/10.3389/fpls.2014.00665.
Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.
Schippers, J.H.M., Schmidt, R., Wagstaff, C., Jing, H.C., 2015. Living to die and dying to live: the survival strategy behind leaf senescence. Plant Physiol. 69, 914–930,. www.plantphysiol.org/cgi/doi/10.1104/pp.15.00498.
Schippers, J.H.M., 2015. Transcriptional networks in leaf senescence. Curr. Opin. Plant Biol. 77–83. http://dx.doi.org/10.1016/j.pbi.2015.06.018.
Schroeder, J.I., Allen, G.J., Hugouvieux, V., Kwak, J.M., Waner, D., 2001. Guard cell signal transduction. Annu. Rev. Plant Physiol. Plant Mol. Biol. 52, 627–658.
Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.
Vandenbussche, F., Vriezen, W.H., Smalle, J., Laarhoven, J.J.J., Harren, F.J.M., Van Der Straeten, D., 2003. Ethylene and auxin control the Arabidopsis response to decreased light intensity. Plant Physiol. 133, 517–527.
Vanderstraeten, L., Van Der Straeten, D., 2017. Accumulation and transport of 1-aminocyclopropane-1-carboxylic acid (ACC) in plants: current status, considerations for future research and agronomic applications. Front. Plant Sci. 8, 38. https://doi.org/10.3389/fpls.2017.00038.
Wang, H., Liu, G., Li, C., Powell, A.L., Reid, M.S., Zhang, Z., et al., 2013. Defenceresponses regulated by jasmonate and delayed senescence caused by ethylene receptor mutation contribute to the tolerance of petunia to *Botrytis cinerea*. Mol. Plant Pathol. 14, 453–469.
Wang, Y., Chen, Y., Ding, L., Zhang, J., Wei, J., Wang, H., 2015. Validation of reference genes for gene expression by quantitative real-time RT-PCR in stem segments spanning primary to secondary growth in *Populus tomentosa*. PLoS One 11 (6), e0157370. https://doi.org/10.1371/journal.pone.0157370.
Wang, Y., Liu, C., Li, K., Sun, F., Hu, H., Li, X., Zhao, Y., Han, C., Zhang, W., Duan, Y., Liu, M., Li, X., 2007. Arabidopsis EIN2 modulates stress response through abscisic acid response pathway. Plant Mol. Biol. 64, 633–644.
Wen, X., Zhang, C., Ji, Y., Zhao, Q., He, W., An, F., et al., 2012. Activation of ethylene signaling is mediated by nuclear translocation of the cleaved EIN2 carboxyl terminus. Cell Res. 22, 1613–1616.
Wilkinson, S., Davies, W.J., 2009. Ozone suppresses soil drying-and abscisic acid (ABA)-induced stomatal closure via an ethylene-dependent mechanism. Plant Cell Environ. 32, 949–959.
Wilkinson, S., Davies, W.J., 2010. Drought, ozone, ABA and ethylene: new insights from cell to plant to community. Plant Cell Environ. 33, 510–525.
Yang, S.F., Hoffman, N.E., 1984. Ethylene biosynthesis and its regulation in higher plants. Annu. Rev. Plant Physiol. 35, 155–189.
Yasumura, Y., Pierik, R., Kelly, S., Sakuta, M., Voesenek, L.A.C.J.K., Harberd, N.P., 2015. An ancestral role for Constitutive Triple Response 1 proteins in both ethylene and abscisic acid signaling. Plant Physiol. 169, 283–298.
Zhang, F., Qi, B., Wang, L., Zhao, B., Rode, S., Riggan, N.D., Ecker, J.R., Qiao, H., 2016. EIN2-dependent regulation of acetylation of histone H3K14 and non-canonical histone H3K23 in ethylene signalling. Nat. Commun. 7, 13018. https://doi.org/10.1038/ncomms13018.
Zhang, F., Wang, L., Qi, B., Zhao, B., Ko, E.E., Riggan, N.D., Chin, K., Qiao, H., 2017. EIN2 mediates direct regulation of histone acetylation in the ethylene response. Proc. Natl. Acad. Sci. U.S.A. 117, 10274–10279.
Zhang, F., Wang, L., Ko, E.E., Shao, K.J., Qiao, H., 2018. Histone deacetylases SRT1 and SRT2 interact with ENAP1 to mediate ethylene-induced transcriptional repression. Plant Cell 30, 153–166.
Zhao, Y., Du, L., Zhang, N., 2013. Sensitivity of prestaining RNA with ethidium bromide before electrophoresis and performance of subsequent northern blots using heterologous DNA probes. Mol. Biotechnol. 54, 204–210.
Zheng, Y., Zhu, Z., 2016. Relaying the ethylene signal: new Roles for EIN2. Trends Plant Sci. 21. https://doi.org/10.1016/j.tplants.2015.11.007.
Zhong, S., Lin, Z., Grierson, D., 2008. Tomato ethylene receptor–CTR interactions: visualization of Never-Ripe interactions with multiple CTRs at the endoplasmic reticulum. J. Exp. Bot. 59, 965–972.