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Aquaporins and Leaf Hydraulics: Poplar Sheds New Light

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To help understand leaf hydraulic conductance (K_{leaf}) modulation under high irradiance, well-watered poplars (*Populus trichocarpa* Torr. & Gray ex Hook and *Populus nigra* L.) were studied diurnally at molecular and ecophysiological scales. Transcriptional and translational modulations of plasma membrane intrinsic protein (PIP) aquaporins were evaluated in leaf samples during diurnal time courses. Among the 15 poplar *PIP* genes, a subset of two *PIP1s* and seven *PIP2s* are precociously induced within the first hour of the photoperiod concomitantly with a K_{leaf} increase. Since expression patterns were cyclic and reproducible over several days, we hypothesized that endogenous signals could be involved in PIP transcriptional regulation. To address this question, plants were submitted to forced darkness during their subjective photoperiod and compared with their control counterparts, which showed that some *PIP1s* and *PIP2s* have circadian regulation while others did not. Promoter analysis revealed that a large number of hormone, light, stress response and circadian elements are present. Finally, involvement of aquaporins is supported by the reduction of K_{leaf} by $HgCl_2$ treatment.

Keywords: Aquaporin • Circadian regulation • Leaf hydraulic conductance (K_{leaf}) • Light • Poplar (*Populus trichocarpa*, *Populus nigra*) • Promoter.

Abbreviations: AQP, aquaporin; g_s, stomatal conductance; HPFM, high pressure flow meter; K_{leaf}, leaf hydraulic conductance; MIP, major intrinsic protein; PIP, plasma membrane intrinsic protein; VPD, vapor pressure deficit.

Introduction

Plant–water relationships are a challenging research topic, but very worthwhile in the context of global climate change. Water uptake by the root system is driven by transpiration (E), which pulls water from the roots to the leaves where it evaporates. Transpiration is the main driving force for water uptake, leading to the establishment of a water potential (Ψ) gradient through the plant. The process is regulated by stomatal aperture opening or conductance (g_s) and driven by the air water vapor pressure deficit (VPD). With a non-limiting water supply, poplar E and g_s are highly and positively linked to the flux of light (Hinckley et al. 1994). Under a limiting water supply, poplar leaf water potential (ψ_{leaf}) and g_s decrease with soil water potential (ψ_{soil}) (Silim et al. 2009, Almeida-Rodriguez et al. 2010). Leaf hydraulic conductance (K_{leaf}), representing the inverse of leaf resistance to water flux, is also modulated by light quality and intensity (Scoffoni et al. 2008, Sellin et al. 2008) in various plant models. The plasma membrane intrinsic proteins (PIPs), members of a plant aquaporin (AQP) subfamily, are suspected to play notable roles in K_{leaf} modulation (Cochard et al. 2007, Voicu et al. 2008) since they have also been shown to influence root hydraulic conductance (Javot and Maurel 2002, Postaire et al. 2009 Vandeleur et al. 2009).

AQPs belong to the ubiquitous major intrinsic protein (MIP) superfamily, a highly conserved family with members ranging in size from 23 to 31 kDa (Gomes et al. 2009). Higher plant AQPs are divided into five main subfamilies based on their sequence similarities (Johanson et al. 2001), among which PIPs and tonoplast membrane intrinsic proteins (TIPs) have both been experimentally shown to increase membrane water permeability (Daniels et al. 1994). The nodulin 26-like intrinsic membrane proteins (NIPs) and the small basic intrinsic proteins (SIPs) constitute the two other MIP subfamilies (Maurel et al. 2008). Recently, X intrinsic proteins (XIPs) were identified in some plant and moss species (Danielson and Johanson 2008, Bienert et al. 2011). Poplar XIPs appear to be plasma membrane-specific AQPs with lower water permeability than TIPs and PIPs (Lopez et al. 2012).

Among the MIP subfamilies, members of the PIP subfamily are historically the most studied because of their location in the plasma membrane and mechanisms of regulation. PIPs form tetramers in which each monomer acts as a functional channel (Chaumont et al. 2005). The PIP subfamily is further divided into two groups: PIP1s and PIP2s. Although residues constituting their selectivity filters are similar, allowing high water

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conductance, PIP1s and PIP2s have different roles (Wallace and Roberts 2004). Nevertheless, all PIP2s expressed in *Xenopus* oocytes have higher water channel activity than PIP1s. AQPs are also associated with mesophyll conductance of CO_2, as demonstrated by the pioneering work of Terashima and Ono (2002) using the AQP inhibitor $HgCl_2$. Direct evidence for their involvement has been obtained in diverse plant species by genetic engineering in tobacco (NtAQP1, Uehlein et al. 2003, Uehlein et al. 2008), rice (HvPIP2;1, Hanba et al. 2004) and Arabidopsis (NtAQP1, Sade et al. 2010; AtPIP1;2, Kaldenhoff 2012, Uehlein et al. 2012).

AQP-mediated water permeability can be modulated through transcription, translation and post-translational modifications and via membrane vesicle trafficking (Maurel et al. 2008). Gene expression can be influenced by environmental conditions and endogenous plant signals, and PIP AQPs have been shown to be regulated by both. For instance AtPIP1;2, shown to increase cell to cell water transport, is regulated by an endogenous circadian rhythm in roots, by blue light in leaves, and by temperature, gibberellic acid, ABA cytosolic pH (Kaldenhoff et al. 1996, Aharon et al. 2003, Tournaire-Roux et al. 2003, Takase et al. 2011, Kuwagata et al. 2012). Previous work carried out on *Samanea saman* (rain tree) showed circadian regulation of leaf PIPs associated with diurnal patterns of water permeability (Moshelion et al. 2002). Tetrameric arrangements of PIP1s and PIP2s could allow an even higher level of AQP activity modulation by regulation of vesicular trafficking (Fetter et al. 2004, Zelazny et al. 2007, Mahdieh et al. 2008) and increased water transport activity (Secchi et al. 2010). Interestingly, inhibitor studies originally showed decreased water permeability in single cells and also in roots and leaves (Wan and Zwiazek 1999, Moshelion et al. 2002, Secchi et al. 2009)

Correlations between leaf-water relations at ecophysiological and molecular levels in the poplar tree model are still poorly understood. To investigate potential links, leaf PIP1 and PIP2 transcripts and proteins in well-watered plants were quantified during light and dark courses while K_{leaf}, g_s and ψ_{leaf} were measured. PIP expression has already been characterized in roots and stems of the *Populus* genus (Marjanović et al. 2005, Hacke et al. 2010, Secchi et al. 2010, Almeida-Rodriguez et al. 2011, Secchi and Zwienuicki 2011, Leng et al. 2012). In addition, recent studies investigated leaf AQP expression (Almeida-Rodriguez et al. 2010) in water-stressed plants. Significantly, little is known of light-dependent PIP modulation in *Populus* leaf tissue.

Our study comprehensively describes light-mediated regulation of PIP transcripts and proteins in leaves of two poplar clones. Furthermore, in light of the conflicting results and conclusions that have been given on the hydraulic functions within various species of plants and the involvement of AQPs, we provide new results for poplar and compile them with available data for the genus. Finally, this work provides a resource for the systematic and detailed investigation of PIP transcripts in poplar, demonstrating success in monitoring 15 PIPs in various hybrids and cultivars including *Populus deltoides*×*P. nigra* (*P. euramericana*), *P. tremula*×*P. alba* and *P. deltoides* (D. Lopez et al. unpublished data).

Results

Leaf hydraulics of *Populus* clones

Leaf hydraulic conductance (K_{leaf}), leaf water potential (ψ_{leaf}) and stomatal conductance (g_s) were measured daily on greenhouse potted *P. trichocarpa* and *P. nigra* clones, one clone each (**Fig. 1**). We observed that the two clones had qualitatively and quantitatively similar ecophysiological behaviors. Under non-limiting water supply and irradiance, both showed relatively conserved hydraulic patterns over time. Both reached

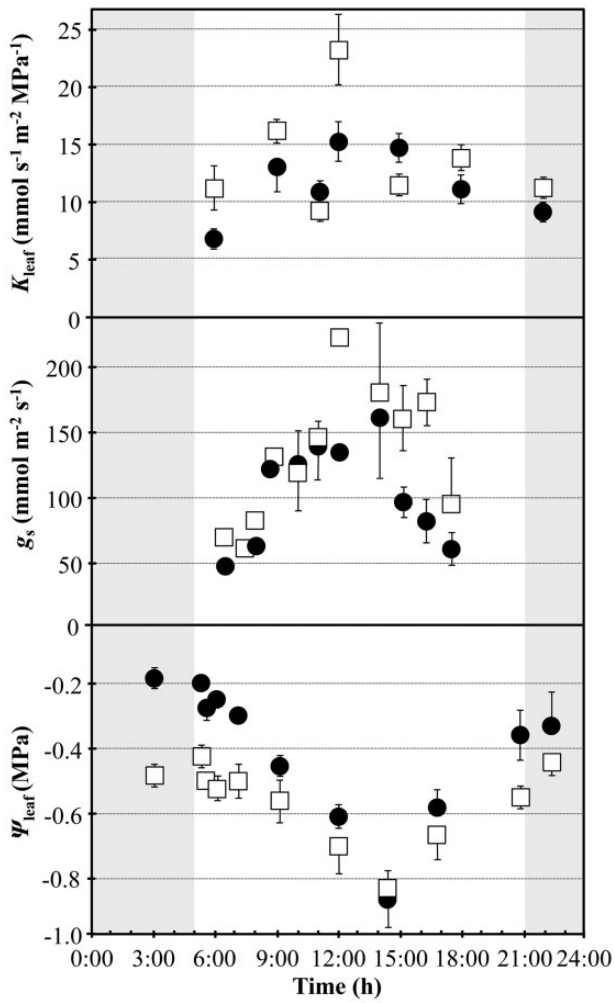

Fig. 1 Diurnal variation of leaf water conductance (K_{leaf}), stomatal conductance (g_s) and leaf water potential (ψ_{leaf}) for *P. nigra* (filled circles) and *P. trichocarpa* (open squares). Time of the day in 24 h format is indicated on the *x*-axis. The white area represents the 16 h photoperiod beginning at 05:00 h and the shaded area indicates the dark period. Results are given as the mean ±SE ($n \geq 6$ for each measurement).

their lowest ψ_{leaf} (−0.83 ± 0.06 MPa for *P. trichocarpa* and −0.81 ± 0.1 MPa for *P. nigra*) between 12:00 h and 14:00 h, which corresponded to midday solar time. Even in non-limiting water supply conditions, *P. trichocarpa* reached a relatively low pre-dawn ψ_{leaf} of −0.48 ± 0.03 MPa, while *P. nigra* pre-dawn ψ_{leaf} was higher at −0.18 ± 0.01 MPa. Both clones also had very similar g_s during the day, with a maximum at midday.

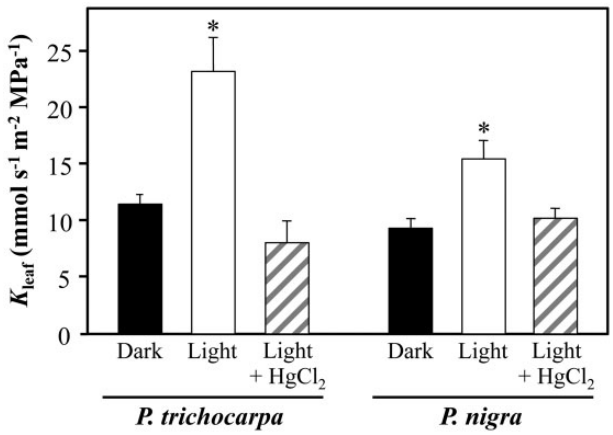

Fig. 2 K_{leaf} of *P. nigra* and *P. trichocapra* grown in normal conditions (white bars), in normal light with 1 h perfusion of 1 mM $HgCl_2$ (hatched bars), and under darkness until midday (filled bars). Inhibitor treatments were carried out at 12:00 h since both clones showed maximum K_{leaf} at this time of the day. Values at 23:00 h are given to compare light-dependent K_{leaf} increase and its relative reduction using $HgCl_2$. Results are given as mean ± SE (n = 14 per clone); asterisks indicate statistically significant differences ($P < 0.05$, Tukey post-hoc test after one-way analysis of variance (ANOVA).

Ramets of the *P. trichocarpa* clone had a greater g_s than the *P. nigra* clone in the afternoon. Using a high pressure flow meter (HPFM) in the greenhouse to measure K_{leaf}, we observed that both clones showed an initial K_{leaf} increase during the morning followed by a decrease at 11:00 h. *Populus nigra* ramets then showed a limited K_{leaf} increase while *P. trichocarpa* ramets showed a sharp increase up to midday. From 06:00 h to 12:00 h, *P. trichocarpa* and *P. nigra* leaves had an average K_{leaf} increase of 107.9% and 120.9%, respectively. From 12:00 h to 23:00 h, *P. trichocarpa* and *P. nigra* leaves experienced a 51.5% and 40.3% K_{leaf} decrease, respectively, reaching values similar to those measured at 06:00 h.

$HgCl_2$, an AQP inhibitor, was used to determine the fraction of K_{leaf} that could be attributed to AQP activity. Leaves were perfused with 1 mM $HgCl_2$ at midday (12:00 h), during the period of maximum water flux. After 1 h of treatment, *P. trichocarpa* and *P. nigra* K_{leaf} was reduced by 66.6% and 27.7%, respectively (**Fig. 2**).

PIP1 and *PIP2* gene expression is regulated diurnally in poplar

Pre-dawn leaves (03:00 h) were used as a reference point for real-time quantitative PCR of diurnal modulation of *PIP* gene expression. The short-term responses of *PIP* expression to light were investigated by sampling leaves 15, 30, 60 and 120 min after the beginning of the photoperiod (05:00 h). Mid- and long-term responses were evaluated by sampling at midday, in the afternoon (18:00 h) and during the following night (21:30 and 23:00 h; **Fig. 3** and **Supplementary Fig. S1** for individual profiles). The poplar genome encodes 15 PIPs, including five PIP1s and 10 PIP2s, which were all included in this experiment.

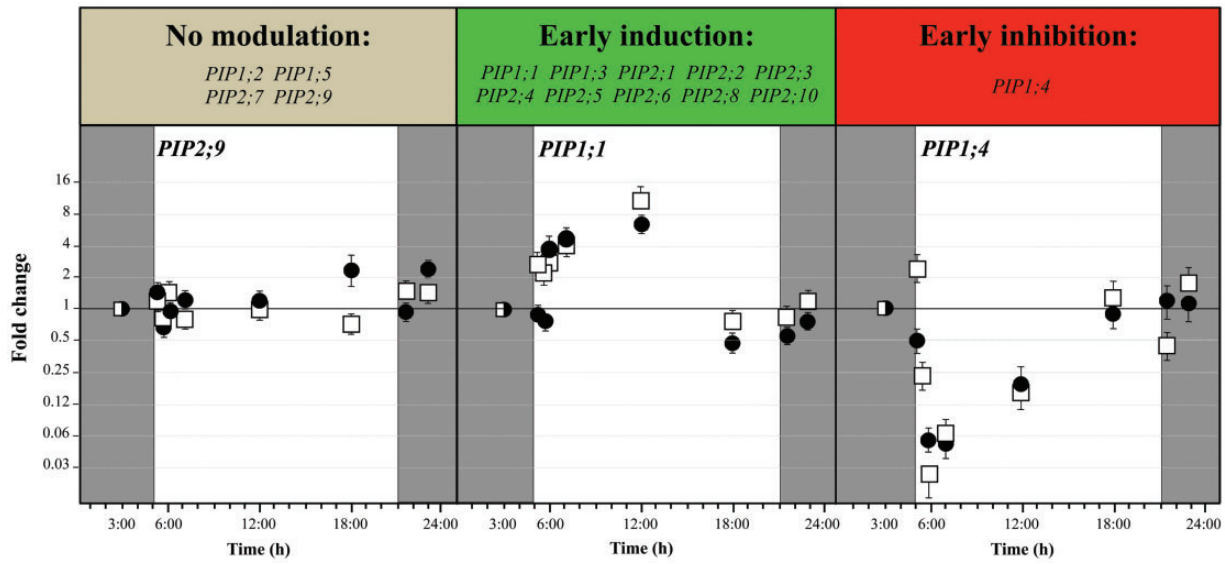

Fig. 3 Poplar *PIP1* and *PIP2* diurnal RNA expression patterns in leaves of *P. nigra* (filled circles) and *P. trichocarpa* (open squares). Time of the day in 24 h format is indicated on the *x*-axis. The white area represents the 16 h photoperiod beginning at 05:00 h and the shaded area indicates the dark period beginning at 21:00 h. Expression levels are given as the relative fold change of each *PIP* transcript compared with the t = 0 (03:00 h) value using the $2^{-\Delta\Delta Ct}$ method. Data are represented as the mean ± SE (n = 3–6). See **Supplementary Fig. S1** for the 15 *PIP* individual profiles.

Interestingly, for most isoforms RNA expression patterns were well conserved between *P. trichocarpa* and *P. nigra* clones. Among PIP1s, *PIP1;1* expression was rapidly and highly up-regulated, reaching a maximum at midday, and then dramatically down-regulated to reach the pre-dawn expression level in the afternoon. The *PIP1;3* mRNA level also increased in the morning but to a lesser extent, and then also decreased in the afternoon. *PIP1;4* expression was strongly repressed starting 30 min after photoperiod onset. Its expression then steadily increased during the afternoon to reach the pre-dawn level. *PIP1;2* and *PIP1;5* levels remained stable in this experiment. Similarly, most PIP2s showed diurnal patterns of transcriptional regulation. Seven (*PIP2;1*, *PIP2;3*–*PIP2;6*, *PIP2.8* and *PIP2;10*) were rapidly induced in the morning for both *P. nigra* and *P. trichocarpa*. *PIP2;7* and *PIP2;9* diurnal modulation was significantly less pronounced, reaching a maximum 2-fold increase by midday. *PIP2;2* expression was slightly modulated in *P. trichocarpa* while in *P. nigra* this isoform was initially induced, followed by a decline with a second peak in the afternoon.

Some minor discrepancies in the overall conservation of diurnal transcription patterns between the two clones could be observed at different time points, more specifically at 18:00 h for *PIP1;5* and for most *PIP2s*. However, physiological patterns were similar for the two clones at this time of the day.

Light effect on leaf hydraulics and PIP expression

To identify whether *PIP* expression patterns were induced by light or by endogenous factors, 'dark plants' were artificially maintained under full darkness until midday. Leaf water potential of dark plants remained steady during the course of the experiment. Midday ψ_{leaf} for dark plants was similar to the pre-dawn value (*P. trichocarpa*, -0.34 ± 0.1 MPa; *P. nigra*, -0.15 ± 0.06 MPa; both $P < 0.001$), probably due to the absence of substantial transpiration. *PIP* expression in dark plants was then compared with that of plants grown under normal light conditions (**Fig. 4**). *PIP1;1* and *PIP1;3* expression was strongly induced in control plants, while dark plants showed a slight

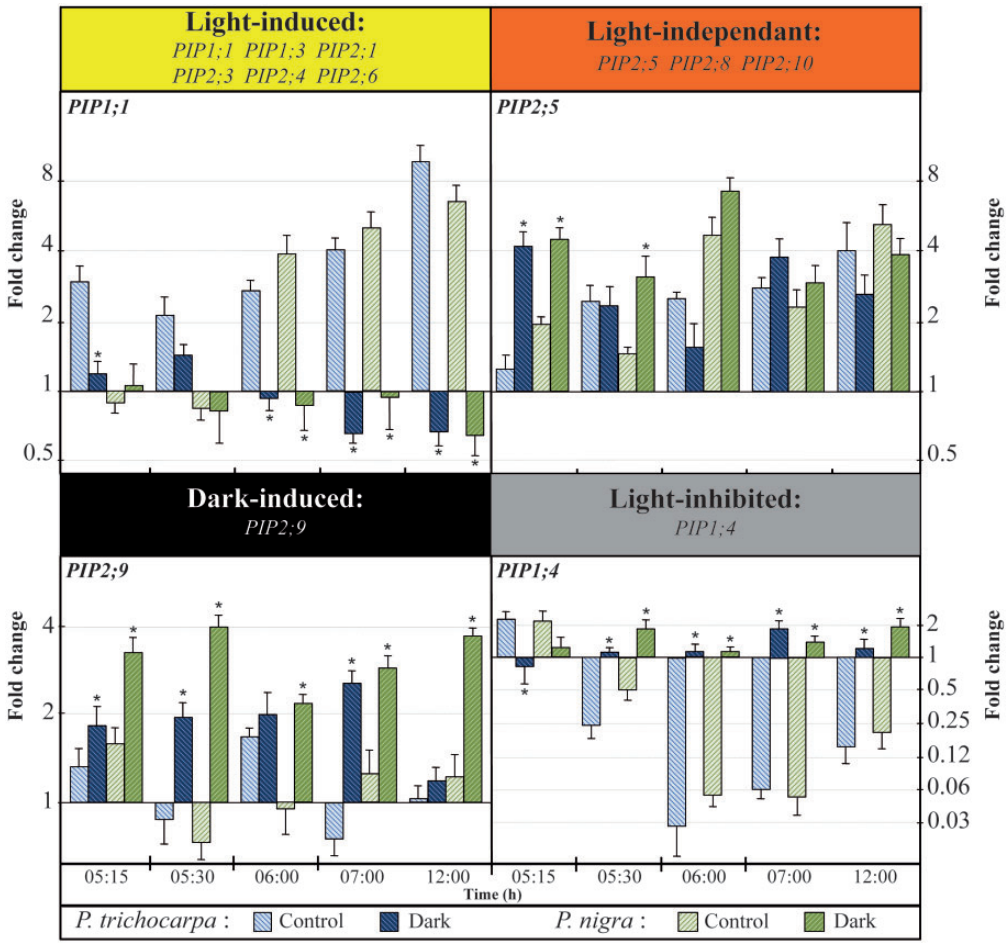

Fig. 4 Leaf poplar *PIP1* and *PIP2* RNA expression patterns in *P. nigra* (green bars) and *P. trichocarpa* (blue bars) subjected to forced darkness (dark-colored bars) compared with their normal light counterparts (light-colored bars). Relative transcript accumulation is given at 15, 30, 60 and 120 min after photoperiod onset (05:00 h) and at 12:00 h. Levels are given as the relative fold change of each PIP compared with the $t = 0$ (03:00 h, pre-dawn) value using the $2^{-\Delta\Delta Ct}$ method. Data are represented as the mean ± SE ($n = 4$); asterisks indicate statistically significant differences ($P < 0.05$, Student's *t*-test). See **Supplementary Fig. S2** for the 15 *PIP* individual profiles.

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variation in relative transcript abundance. *PIP1;4*, strongly down-regulated during the morning in control plants, showed little variation in dark plants. *PIP2;1*, *PIP2;3*, *PIP2;4* and *PIP2;6* were more strongly induced in control plants, although *P. nigra PIP2;4* mRNA was more abundant in the late morning. *PIP2;5*, *PIP2;8* and *PIP2;10* were up-regulated in dark plants as in control plants. *PIP1;5*, *PIP2;2* and *PIP2;7* transcript levels did not vary diurnally with either treatment. Interestingly, *PIP2;9* appears to be a dark-induced isoform, which is also indicated by the relatively high expression levels found in the evening (**Fig. 4**; see **Supplementary Fig. S2** for individual profiles).

PIP proteins accumulate during the day

Ultimately, *PIP* transcripts will be translated into proteins that will act as functional channels once trafficked to plasma membrane. Since the levels of *PIP* transcripts and proteins are not always correlated (Hachez et al. 2012), it is of great interest to evaluate the levels of the related proteins. Leaf samples from the two clones growing in normal light conditions and in forced darkness were harvested at pre-dawn (03:00 h) and at midday (12:00 h ±Light). **Fig. 5** shows in *P. trichocarpa*, and to a lesser extent in *P. nigra*, an accumulation between 03:00 h and 12:00 h (+ Light) of a single PIP1 product at ~30 kDa, which corresponds to the predicted size of poplar PIP1s. For both species, the amount of protein recognized by the PIP1 antibody was lower at 03:00 h or 12:00 h −Light than at 12:00 h +Light. PIP2 antibody labeling revealed proteins corresponding to the size of PIP2 monomers and dimers (~30 and ~58 kDa, respectively; Bienert et al. 2012). Compared with 03:00 h and 12:00 h ± Light, levels of PIP2s did not change in *P. trichocarpa* but showed a peak in *P. nigra* in the 12:00 h +Light sample. Observed levels of apparent PIP dimers followed the abundance of the monomer during these experiments.

Sequence analysis

PIP family members share very conserved amino acid sequences in poplar. Globally, the 15 PIPs have 72.2% pairwise identity. The 10 PIP2s have 77.8% pairwise identity while PIP1s show a higher homology with 86.3% pairwise identity. The average amino acid similarity between the products of *PIP1;1* and *PIP1;3* is very high (95.5% pairwise identity), suggesting they are duplicates or paralogs. *PIP2;5* and *PIP2;10* have highly conserved sequences (98.3% pairwise identity) and also show similar transcriptional modulation in our experiments (**Figs. 3**, **4**). The proximity of the two genetic loci (Potri.006G128000 and Potri.006G128200) further supports the hypothesis of a gene duplication event. Such gene clustering is not unique in the PIP family, and other highly homologous PIP2s are located in proximity to each other (**Supplementary Table S1**), although *PIP2;8* and *PIP2;9*, are largely divergent (65.9% pairwise identity, respectively; Potri.005G109300 and Potri.005G109200). *PIP1;4* (Potri.06G098100) is located close to a *PIP1* pseudogene (Potri.06G097900) coding for a premature stop codon. Duplication events usually lead either to loss of function for one of the duplicates, neo functionalization or function overlapping (Conant and Wolfe 2008), and such events have been documented for poplar *XIPs* (Lopez et al. 2012).

Studying a fully sequenced model plant allows the characterization of non-expressed sequences, particularly those forming promoter domains. We analyzed ~1.5 kb upstream of the initiation codons of the 15 poplar *PIPs*, two *Arabidopsis thaliana PIPs* (*AtPIP1;2* and *AtPIP2;1*) and four *Zea mays PIPs*

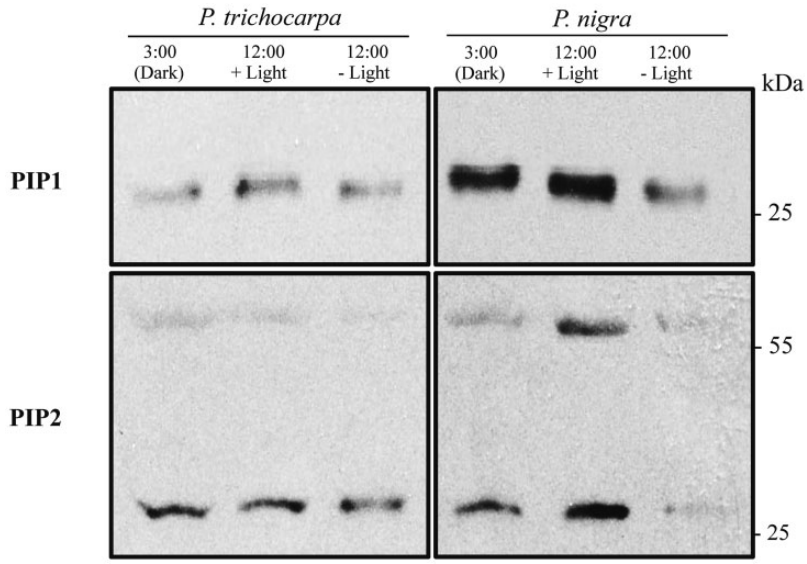

Fig. 5 Western blot of microsomal proteins from leaves of *P. trichocapra* and *P. nigra* separated by SDS–PAGE and immunolabeled using PIP1 or PIP2 antisera. Protein was extracted from plants at pre-dawn (03:00 h), midday (12:00 h + Light), and midday on plants submitted to continuous darkness (12:00 h − Light). PIP2 antisera also labeled probable aquaporin dimers of ~60 kDa.

Table 1 Putative *cis*-acting sequences detected by PLACE signal scan on an ~1.5 kb proximal promoter region of 15 *P. trichocarpa*, two *A. thaliana* and four *Z. mays* PIPs.

		Populus trichocarpa																*Arabidopsis thaliana*		*Zea mays*			
		PIP1.1	*PIP1.2*	*PIP1.3*	*PIP1.4*	*PIP1.5*	*PIP2.1*	*PIP2.2*	*PIP2.3*	*PIP2.4*	*PIP2.5*	*PIP2.6*	*PIP2.7*	*PIP2.8*	*PIP2.9*	*PIP2.10*	*AtPIP1;2*	*AtPIP2;1*	*ZmPIP1;2*	*ZmPIP1;5*	*ZmPIP2;1*	*ZmPIP2;5*
Light	EBOXBNNAPA (S000144)	8	2	8	10	12	8	6	6	10	2	12	4	10	10	4	10	2		10		12
	GATABOX (S000039)	12	13	11	7	12	10	5	12	3	10	8	8	7	18	8	12	15	4	10	12	5
	GT1CONSENSUS (S000198)/GT1CORE (S000125)	11	9	18	20	13	6	12	23	10	8	14	6	11	20	6	13	14	3	7	16	9
	IBOX (S000124)/ IBOXCORE (S000199)/ IBOXCORENT (S000199)	3	10	3	3	5		3	9	1	7	3	3	3	10	3	9	4	2	5	6	4
	INRNTPSADB (S000395)	5		2	3	4	3	1	12	2		1		2		1	1	2	1		6	1
	TBOXATGAPB (S000383)	1					1				2	1	2			1		1				1
Light/PhyA	SORLIP1AT (S000482)/ SORLIP2AT (S000483)/ SORLREP3AT (S000488)/SORLIP5AT (S000486)	3	2	3	5	1	2	3		3	1	5	2		1	2	2	3	2	10	2	6
Phytochrome	REALPHALGLHCB21(-S000362)/ REBETALGLHCB21 (S000363)		1	1				3	1	1	1	2		2	5	4		2			1	
	Total	43	36	45	48	47	30	30	62	29	30	44	25	33	59	25	47	41	12	42	42	38
Light/ circadian	−10PEHVPSB (S000392)	1	3			1	2			1	1	1	1	2								2
	CIACADIANLELHC (S000252)	2		1	2	1	1		5		1		1	1	2					1	2	
	Total	3	3	1	2	2	3		5	1	2	1	2	3	2					1	2	2
ABA	ABRELATERD1 (S000414)/ ACGTABREMOTIFA2-OSEM (S000394)/ DPBFCOREDCDC3 (S000292)	2	3	1	3	3	4	1	1	1	2	7	3	3	1	2		1	1	4	1	6
ABA/Ca2+	ABRERATCAL (S000507)						1	1	1			2	1	1	1							3
ABA/WS	DRE2COREZMRAB17 (S000402)	1		1	1	1		1			1	1						1	1	1		1
MYB/ABA/ water stress	MYB1AT (S00408)/ MYB2AT (S000177)/ MYBATRD22 (S000175)/MYBCORE (S000176)/ MYB2CONSENSUSAT (S000409)	10	2	9	3	11	10	14	4	5	12	4	5	5	10	2	3	5	3	9	3	14
MYC/ABA/ water stress	MYCATERD1 (S000413)/ MYCATRD22 (S000174)/ MYCCONSENSUSAT (S000407)	10	2	10	12	18	12	8	10	10	2	12	4	12	16	6	14	4		10	11	18
Water stress	CBFVH (S000497)	3		1	1	1						1	1					2	2	1		1
	Total	26	7	22	20	34	27	25	16	16	17	27	14	21	28	10	17	13	7	25	15	43

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(*ZmPIP1;1*, *ZmPIP1;5*, *ZmPIP2;1* and *ZmPIP2;5*), all of which are well characterized and known to have circadian regulation patterns (Lopez et al. 2003, Takase et al. 2011). In silico analysis showed that the regions upstream of poplar PIPs had putative response elements for virtually all plant hormones, including ABA, salicylic acid, auxin, cytokinins, gibberellins, jasmonate, ethylene and sugars, as well as for abiotic stress responses such as wounding, dehydration and cold. Since our interest is in diurnal leaf water relations, we focused on promoter elements putatively associated with drought and light responses (**Table 1**). Poplar *PIPs* have a substantial number of ABA-responsive elements (ABREs) as well as MYB and MYC response elements associated with water stress and drought. Numerous putative light response units (LRUs) were also found, especially in *PIP2;3*, which is in line with our results showing that all poplar *PIPs* respond to light.

Discussion

The aim of this work was to characterize the diurnal modulations of leaf water permeability in poplar and the involvement of AQPs in this process. Before commencing an analysis of poplar *PIP1* and *PIP2* gene and protein expression, it was necessary to identify these correctly using the current PIP nomenclature. In previous studies of poplar AQPs, various overlapping AQP nomenclatures were created as occurred in *Arabidopsis* and other plants. In our work we use the original poplar PIP nomenclature presented by Secchi et al. (2009), which is consistent with the seven sequences originally released by Marjanović et al. (2005). **Supplementary Table S1** lists the various aliases for the 15 poplar *PIP* genes along with their corresponding genomic loci in the newly released *P. trichocarpa* v3.0 annotation.

Diurnal regulation of poplar leaf hydraulics

To ensure that our results were informative, we generated control plants grown with non-limiting water and light availability. Regardless of any treatment, organisms used as experimental controls adjust themselves physiologically to their environmental conditions, particularly in response to their circadian rhythms. Therefore, instead of using our untreated plants as simple negative controls, their physiological responses were carefully analyzed. Leaf water potential was measured to ensure plants had a sufficient water supply, to avoid stomatal closure and concomitantly reduced leaf water flow and transpiration. In our experiments, when the water supply was non-limiting the result was a relatively high midday ψ_{leaf} and an increase in diurnal K_{leaf}. Water-stressed plants tend to decrease the former and inhibit the latter (Aasamaa et al. 2005). Pre-dawn ψ_{leaf} values obtained in our experiments are in agreement with what is typically observed in non-stressed greenhouse poplars grown in pots (Cocozza et al. 2010, Awad et al. 2010, Larchevêque et al. 2011). These values were also close to those reported for plants grown in low to high irradiance (Almeida-Rodriguez et al. 2011). The relatively low pre-dawn ψ_{leaf} observed with *P. trichocarpa* could be explained by relatively high nightly transpiration, which has been reported for this species in the field (Snyder et al. 2003) and supported by the observation of substantial night sap flow in *P. trichocarpa*×*P. deltoides* trees (Meiresonne et al. 1999).

Despite relatively similar hydraulic characteristics while not under stress, our two poplar clones differ remarkably in their susceptibility to xylem cavitation. *Populus nigra* and *P. trichocarpa* are one of the least and one of the most susceptible species in the genus, respectively. Xylem embolism susceptibility is generally associated with decreased xylem conductivity. Given a sufficient water supply, having K_{leaf} positively linked with g_s diminishes the tension imposed on the water column and allows ψ_{leaf} to be maintained above the threshold value below which cavitation risks increase (Tyree et al. 2005).

Experimental evidence of diurnal variations in K_{leaf} of various plant species led to conflicting conclusions. Measured *in situ*, our two poplar clones diurnally increased K_{leaf} as do *Quercus macrocarpa* (Voicu and Zwiazeck 2011), *Helianthus annuus* (Tsuda and Tyree 2000), *Platanus orientalis* and *Aleurites moluccana* leaves (Lo Gullo et al. 2005). These results contrast with either steady or declining values in other species (*Pinus ponderosa*, *Pinus nigra*, *Castanopsis chrysophylla* and *Pieris japonica*) as reviewed in Johnson et al. (2009, 2012). This scenario suggests that poplar can increase bulk leaf water permeability in response to evaporative demand when water availability is not limiting, as hypothesized by Tsuda and Tyree (2000). It is also consistent with previous studies that have shown increased K_{leaf} with high irradiance in various tree species (Sack et al. 2002, Cochard et al. 2007, Scoffoni et al. 2008, Voicu et al. 2008, Ben Bâaziz et al. 2012).

Unlike the apoplastic component of K_{leaf}, regulation of the cell to cell pathway for water involves membrane permeability, potentially via AQPs. However, it is difficult to link K_{leaf} to specific AQP isoforms and expression patterns. While no parallel was found between the expression of four *PIPs* and the K_{leaf} response in bur oak (Voicu et al. 2009), we recently demonstrated an increase in K_{leaf} upon leaf illumination in *Juglans regia*, *Fagus sylvatica*, *Quercus robur* and *Populus tremula* that correlated with the differential regulation of *PIP1* and *PIP2* expression, depending on the species (Ben Bâaziz et al. 2012).

Roles of PIPs in plant water relations

PIP AQP expression appears to follow species-specific patterns that are dependent on environmental conditions. During water stress, AQP transcription is either up-regulated (*Nicotiana glauca* leaves, stem and roots; Smart et al. 2001, Hachez et al. 2012) or down-regulated (*A. thaliana* rosette; Alexandersson et al. 2005). While double antisense transgenic *A. thaliana* with reduced *PIP1* and *PIP2* levels showed no difference in g_s, transpiration, ψ_{leaf}, K_{leaf} and K_{root} under non-limiting water supply or water deficit, drought recovery after rewatering was faster for wild-type plants than for transgenic plants (Martre

et al. 2002). This strongly suggested that PIPs play a role in embolism refilling (Sakr et al. 2003, Secchi and Zwieniecki 2010, Perrone et al. 2012). Subsequent water stress experiments conducted on different poplar clones have clarified the involvement of AQPs. Overall, a relative increase in PIP content was found in roots, stems and leaves of stressed plants (Almeida-Rodriguez et al. 2010, Secchi and Zwieniecki 2010, Almeida-Rodriguez et al. 2011, Leng et al. 2012). It was hypothesized from stem expression patterns that *PIP1;1* and *PIP1;3* could play a role in embolism repair in *P. trichocarpa* (Secchi and Zwieniecki 2010). In *P. alba*×*P. glandulosa* stems and *P. balsamifera*×*P. simonii* leaves, the orthologs of these two genes are also induced in plants subjected to water stress and recovery, further supporting the importance of the two genes in water stress responses (Almeida-Rodriguez et al. 2010, Leng et al. 2012). We also observed a substantial increase in the transcription of these genes at midday. Since the leaves were de-petiolated after water potential measurements and prior to gene expression assays, the observed modulations only involve lamina parenchyma and vessels, and hence do not relate to possible responses to diurnal petiole embolism recovery (Zufferey et al. 2011, Perronne et al. 2012). *PIP2;5* and *PIP2;10* orthologs, previously identified in *P. tremula*×*P. tremuloides*, *Q. macrocarpa* and *J. regia*, are also thought to play a central role in leaf water relations (Marjanović et al. 2005, Cochard et al. 2007, Voicu et al. 2009, Ben Bâaziz et al. 2012). The daily variation in K_{leaf} experienced by the two poplar clones is associated with a substantial water flux capacity that must be supported by an accordingly high fluid reservoir in order to prevent hydraulic failure (Blackman and Brodribb 2011, Muries et al. 2011). These reservoirs remain to be spatially and temporally characterized.

Light and circadian regulation of poplar PIPs

Only a few studies report plant *AQP* promoter structure (Kaldenhoff et al. 1996, Yamada and Bohnert 2000, Li et al. 2009, Tungngoen et al. 2009). Our analysis of poplar *PIP* promoters revealed a large number of hormone, stress and light response elements (**Table 1**). Comparative analysis of Arabidopsis and maize *PIP* promoters showed that they also contain a large number of light, ABA and water stress response elements. *Cis*-elements putatively involved in circadian regulation, present in virtually all poplar *PIP* genes, did not correlate with all results arising from forced darkness. These *cis*-elements are also not found in *Arabidopsis PIP* genes and *ZmPIP1;1* promoters. We hypothesize that other *cis*-elements, as yet uncharacterized, are involved in *PIP* circadian regulation.

Our experiments with dark-morning plants showed that most of the variations in *PIP* expression are different from those in plants growing in normal light conditions, suggesting that they are independent of circadian regulation and associated with events occurring in full sunlight (i.e. high water flow, embolism recovery, CO_2 mesophyll conductance). *PIP1;1* and *PIP1;3* expression was induced in roots in response to increased evaporative demand in *P. trichocarpa*×*P. deltoides*. *PIP1;1* was also found to be induced in response to hormones and various abiotic stresses in leaves and cell cultures (Bae et al. 2011). Our experiments suggest a greater role for *PIP1;1* and *PIP1;3* in the leaf light response.

Several PIP2 isoforms merit special attention. *PIP2;5* was expressed independently of the presence or absence of light and could be under circadian regulation in leaves (**Fig. 4**). Similarly, the expression of *PIP2;8* is to some extent also under endogenous regulation. In *P. deltoides* leaves, circadian expression of *PIP1;1* and *PIP2;6* was reported (Matsubara et al. 2006). In our clones, only *PIP2;6* was notably induced when plants were subjected to forced darkness, and not *PIP1;1*. However, we can not determine unequivocally whether the differences in *PIP* expression between normal and dark-morning plants are induced by light as a signal per se, or are a consequence of the transpiration triggered by the photoperiod. Having said that, the earliest time points of the kinetics (i.e. 15 and 30 min) are not subject to controversy since the greenhouse VPD is not statistically different between pre-dawn and up to 1 h after the beginning of the photoperiod (0.59 kPa, $P > 0.001$). Circadian variations in the expression of *AQP* genes and proteins have already been demonstrated in various plant models in both roots (*Z. mays*, Lopez et al. 2003, Hachez et al., 2012; *A. thaliana*, Takase et al. 2011) and leaves (*Z. mays*, Hachez et al. 2008), and were consistently associated with increased water permeability.

The water channel activities of poplar PIPs have been functionally characterized by heterologous expression in *Xenopus* oocytes (Secchi et al. 2009, Almeida-Rodriguez et al. 2010, Secchi and Zwieniecki 2010). When expressed alone, PIP1;1 does not increase the water permeability of the oocyte membrane whereas all PIP2s do. However, the co-expression of PIP1;1 and PIP2;4 leads to a greater water permeability than PIP2;4 expressed alone (Secchi and Zwieniecki 2010). This synergistic interaction between PIP1s and PIP2s has been reported in various species (Fetter et al. 2004, Vandeleur et al. 2009, Bellati et al. 2010). However, the functional relevance of these hetero-oligomers remains to be established in planta.

Role of PIP proteins in K_{leaf}

In *A. thaliana*, both rosette water conductance (K_{ros}) and AQP mRNA expression are under circadian regulation; furthermore, K_{ros} is sensitive to AQP inhibitors (Postaire et al. 2010). The AQP inhibitor $HgCl_2$ has been reported as a K_{leaf} inhibitor in a wide range of plants (*Helianthus annuus*, Nardini and Salleo 2005; *Glycine max*, Sadok and Sinclair 2010; *P. tremuloides*, Voicu and Zwiazek 2010; *A. thaliana*, Shatil-Cohen et al. 2011). Furthermore, this inhibitor was found to reduce the water permeability (P_f) of oocytes expressing poplar PIP2 AQPs (Secchi et al. 2007, Secchi et al. 2009). In our work described here, $HgCl_2$ induced a decrease in K_{leaf} for leaves of both *P. nigra* and *P. trichocarpa* to levels comparable with those of dark leaves, showing the involvement of mercury-sensitive components such as AQPs in high diurnal K_{leaf} variations.

Immunoblots using promiscuous PIP1 and PIP2 antibodies showed differences in PIP protein levels in microsomal fractions

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from plant leaves taken pre-dawn, at midday and at midday in dark-morning plants. Since these antibodies recognize multiple PIP1 or PIP2 isoforms, our experiments only allow for the observation of trends in levels of PIP1s and PIP2s. However, both anti-PIP1 and anti-PIP2 targets were less abundant in *P. nigra* dark-morning plants than in midday or pre-dawn control plants. Levels of *P. trichocarpa* PIP1 and PIP2 proteins appeared to be less subject to modulation, which could be associated with a less tightly regulated water exchange in the leaves of this species. Nevertheless, PIP2 proteins accumulated in response to light, for *P. nigra* and to a lesser extent for *P. trichocarpa*. Taken together, our immunolabeling results suggest that PIP1 and PIP2 proteins are subject to diurnal turnover in poplar leaves, and these results correlate with our observations of *PIP* transcript levels. Interestingly, previous work showed a lack of correlation between RNA and protein levels (Muries et al. 2011, Hachez et al. 2012). Given the metabolic cost of de novo protein synthesis, it remains intriguing why poplars promote such high transcription and translation levels of PIP aquaporins diurnally instead of regulating them at the protein level (e.g. protein activity, vesicle trafficking); more information needs to be obtained about the stability and localization of these proteins.

Conclusion

PIP AQP expression in *P. trichocarpa* and *P. nigra* follows complex patterns, not only in response to light but also in response to other endogenous factors (**Figs. 4**, **5**). Increased expression levels during the day could therefore result in an increase in water permeability to supply high K_{leaf} while the specifically induced expression of *PIP2;9* in forced darkness could occur as a compensatory mechanism. Based on our observations, we hypothesize that PIPs are regulated via fine adjustments in the levels of transcripts and proteins in response to varying environmental conditions.

Water flux in leaves of *P. nigra* and *P. trichocarpa* is very similar, both at the ecophysiological level and also qualitatively and quantitatively at the molecular level when looking at diurnal and forced darkness expression patterns. The two species diurnally increase leaf water permeability in a similar fashion when grown with a non-limiting water supply. Our results also show that non-stressed plants experience concomitant variations in their AQP levels. Under control conditions, it is striking how well conserved the expression patterns of the PIP1 and PIP2 orthologs are between the two species. This remarkable conservation can reasonably be associated with the conservation of underlying regulatory mechanisms that are fundamental to the *Populus* genus.

Our results indicate that in poplar, high levels of K_{leaf} under light are likely to involve both PIP1 and PIP2 aquaporins. Given that water and CO_2 fluxes are linked to the photosynthetic process and that AQPs serve to facilitate the movement of these molecules, the tissue-specific locations (e.g. parenchyma, stomata and vascular-associated cells) and contributions of each PIP1 and PIP2 isoform remain to be determined.

Materials and Methods

Plant material and experimental design

Ramets of *P. trichocarpa* (Torr. & Gray ex Hook) clone INRA 101-74 and *P. nigra* L. clone INRA 71072-501 were provided by Dr. Catherine Bastien (INRA, Orléans, France). Homogenous 25 cm long cuttings were planted in 20 liter pots filled with commercial substrate (40% black, 30% brown and 30% blond peat moss, pH 6.1, DUMONA-RN 75-3851, Arandon, the Netherlands) and grown in a controlled-environment greenhouse (Blaise Pascal University, Clermont-Ferrand, France) under a 16 h light/8 h dark photoperiod, at 18/22°C (night / day), with the relative humidity set at 70 ± 10%. When incoming sunlight in the greenhouse was below 350 µmol m^{-2} s^{-1} (dawn and dusk), photosynthetic photon flux was maintained using 400 W Master son-T Pia Hg-Free lamps (Phillips). Pots were automatically watered by drip irrigation, maintaining daily field capacity. To achieve forced darkness, an enclosure was placed over four plants of each clone in the greenhouse and leaves were sampled behind a curtain to shield plants from illumination. Statistical analyses were conducted using the R software package (http://www.R-project.org/, R Development Core Team).

Leaf water potential, stomatal conductance and leaf water conductance

Leaf water potential (ψ_{leaf}) was determined using a Scholander pressure chamber (PMS Instruments) at pre-dawn as a proxy of soil water potential, and during diurnal time courses. For each biological replicate, ψ_{leaf} values were obtained by averaging three successive measurements on leaves of different plants. Leaves were depetiolated and immediately frozen in liquid nitrogen for subsequent expression assays. Stomatal conductance (g_s) was recorded using a LI-1600 leaf porometer (Li-Cor). Leaf hydraulic conductance was measured in the greenhouse by the HPFM method (Cochard et al. 2007). Briefly, deionized degassed water was pushed into the petiole under positive pressure (P; MPa) of an excised leaf kept underwater to prevent transpiration and maintain temperature at 22°C. The water flow (F; mmol s^{-1}) was recorded with a computer connected to the HPFM apparatus. Leaf hydraulic conductance (K_{leaf}, mmol s^{-1} m^{-2} MPa^{-1}) was calculated as $K_{leaf} = F/(P \times LA)$, where LA is the leaf area (m^2) measured using an ImageJ macro written by Eric Badel (INRA) after scanning ($n = 49$ for each clone). Inhibitor experiments were carried out at midday when K_{leaf} values were the highest. Using HPFM as previously described in Cochard et al. (2007), deionized water with 1 mM $HgCl_2$ (Sigma-Aldrich) was pushed for 1 h at 0.2 MPa into petioles of illuminated leaves and the relative reduction in K_{leaf} was calculated at the end of each measurement ($n = 14$ per clone).

RNA isolation and PIP gene expression kinetics

Total RNA from depetiolated leaves was extracted according to Chang et al. (1993). First-strand cDNA was then synthesized from 1 µg of total RNA using SuperScript III (Invitrogen)

following the manufacturer's instructions. Real-time PCR was performed using a MyiQ instrument (Bio-Rad) with MESA GREEN qPCR MasterMix Plus (Eurogentec) containing 2 µl of 20-fold diluted cDNA. PCR was started with an initial denaturation at 94°C for 3 min, followed by 40 cycles of amplification [94°C for 20 s, then 54°C (*PIP1* genes) or 58°C (*PIP2* genes) for 20 s, and then 72°C for 20 s]. PCR efficiency was 100 ± 2% for all primer pairs and specificity was checked using melting curves. The threshold cycle for each reaction (Ct) was determined using the Bio-Rad iCycler iQ v2.0 software. Gene expression was measured according to Livak and Schmittgen (2001) and determined as the fold change of an isoform at a given time point relative to its expression at the initial pre-dawn time point (t_0 = 03:00 h). The normalization of target gene expression was achieved using the software application BestKeeper v1 (http://www.gene-quantification.info; Pfaffl et al. 2004), first to determine the most suitable reference genes from nine widely used housekeeping genes (Czechowski et al. 2005, Xu et al. 2011), and then to estimate a BestKeeper Index, which is the geometric mean of the most stable housekeeping genes and used as a calibrator. The reference genes selected (*Actin1* Potri.001G309500, *SAND* Potri.009G014400, *TIP41-like* Potri.009G093200, *UP1* Potri.002G127700 and *EF1-α* Potri.010G309500) were chosen from different protein families in order to reduce the possibility of co-regulation. *PIP* primers were designed in order to be specific to a given isoform but usable on the most poplar species possible. To do so, all available PIP expressed sequence tags (ESTs) available for *Populus* (taxid 3684) were retrieved using BLASTn (Altschul et al. 1997) and aligned with ClustalW (Thompson et al. 1994) using the *P. trichocarpa PIP* sequences as references. Conserved regions between orthologs in the genus were targeted for primer design. All primers used for this study were designed with the Primer3plus application (http://www.bioinformatics.nl/primer3plus, (Untergasser et al. 2007) and are listed in **Supplementary Table S3**.

Microsomal fraction isolation, protein separation and immunoblotting

Depetiolated leaves from pre-dawn (03:00 h) and midday (12:00 h ±Light) plants were flash-frozen and pulverized in liquid nitrogen. Microsomal fractions were obtained by ultracentrifugation of the recovered laminas as described in Nilsson et al. (2010). Protein concentrations were determined using a Bradford assay (Sigma-Aldrich). A 10 µg aliquot of each microsomal fraction was separated by 12% acrylamide SDS–PAGE and then transferred to a nitrocellulose membrane. To ensure homogenous loadings and efficient transfers, gels and membranes were stained using Coomassie blue and Ponceau S solutions (Bio-Rad), respectively. Membranes were blocked using 5% skim milk and incubated for 1 h at room temperature with peptide antibodies showing broad affinity either for PIP1 proteins (GKEEDVRVGANKFPERQPIGTSA, AS09487; Agrisera) or for PIP2 proteins (AKDIEASGPEAGEFSAKD, provided by Dr. François Chaumont) reported to cross-react with tamarack PIP2 (Calvo-Polanco et al. 2012). Putative epitopes on *Populus* PIP1 and PIP2 proteins are indicated in **Supplementary Table S2**. Membranes were then incubated with an anti-rabbit IgG secondary antibody coupled to horseradish peroxidase (HRP; Southern Biotech) for 2 h. Protein–antibody complexes were detected using the Immobilon Western HRP system (Millipore).

Promoter analysis

Promoter sequences of poplar *PIP* genes, *AtPIP1;2* (At2g45960), *AtPIP2;1* (At3g53420), *ZmPIP1;1* (X82633), *ZmPIP1;5* (AF326489), *ZmPIP2;1* (AF326491) and *ZmPIP2;5* (AF130975), were retrieved by searching JGI databases using Phytozome v8.0 (http://www.phytozome.net) and exporting ~1.5 kb of genomic sequence upstream of the initiation codon. Retrieved sequences were analyzed for putative *cis*-acting sequences using the Plant Cis-acting Regulatory DNA Elements (PLACE) signal scan software package (http://www.dna.affrc.go.jp/PLACE/signalscan.html, Higo et al. 1999).

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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