Transcriptional rearrangement by plant growth-promoting rhizobacteria in priming drought tolerance in plants

by

Wenshan Liu

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Approved by

Sang-wook Park, Assistant professor of Plant Pathology
Joseph Kloepper, Professor of Plant Pathology
Fernando Biase, Assistant professor of Animal Science

Abstract

In recent decades, the scarcity of water, referred to as drought, has become one of the most main problems in agriculture throughout the world. Especially since recent increases in human population, and the global climate change, an urgent need has been emerged to develop novel, effective and sustainable strategies such as generating new crop cultivars that confer tolerance or resistance towards drought. Thus, as an initial step, this study aims to uncover generic principles in the heightened states of drought tolerance (or resistance) in Arabidopsis by *i*) establishing plant growth-promoting rhizobacteria (PGPR) as biostimulants for priming drought tolerance (or resistance), *ii*) determinng the pattern change of PGPR-responsive transcripts, and *iii*) discerning genes directely associated with drought tolerance (or resistance) from a list of genes, associated with plant growth and/or responsive to drought and other environmental stresses. Here our new qualitative and high-throughput quantitative analyses both agreed that selective PGPR strains in the species of *Panebacillus polymyxa* and *Bacillus amyloliquefaciens* can prime drought tolerance in Arabidopsis, and soybean plants. The priming occurs in parallel to the rapid induction of PGPR-inducible genes (PIGs) which are associated with abscisic acid (ABA) and jasmonic acid (JA) signaling pathways. Interestingly, a subset of ABA-dependent PIGs are known as 'memory' genes in dehydration, suggesting that PGPR hijack and trigger drought-induced systemic resistance (ISR). However, PIGs also include other ABA-responsive genes that are induced by drought and other abiotic stresses such as cold temperature (i.e., *Low temperature induced 79*, also called *RD29A*, gene) but reported as non-

memory gene. Hence, we conclude that an intricate metabolic network is involved in the PGPR-induced priming of drought tolerance which also related to other stress acclimation processes (e.g., cold, tissue injury and UV damage) as well as disease resistance (e.g., microbial and insect infections), which are agreed with the known benefactory effects of PGPR towards various aspects of plant growth, development and survival.

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Introduction

Drought is a period that water supply and contents are lower than the average precipitations. In the past few decades, drought has become one of the most important problems that significantly reduce the global food supply. Recently, drought-induced losses in crop yields have surpassed losses from all other causes combined (Blum, 1988). Hence, there is an urgent need for development of effective and sustainable drought management programs, such as new resistance cultivars. However to reach this aim, we need much more knowledge about the stress physiology of plants during drought and high temperatures. Therefore, furthering our understanding of cellular and metabolic changes in plants during drought stress will provide molecular/genetic principles that will assist genetic engineering or molecular breeding research to upgrade plant defense capacities and improve yield and survival.

Plants have evolutionarily developed a layer of cellular mechanisms, transitioning gene expressions that trigger stomatal closure and limits respiration under drought stress (Taii and Ohasumi, 2002). The caveat is that these reactions result in a delay of plant growth and damage to plant cells and tissues (Fang and Xiong, 2015). Thus, a number of research studies have focused mainly on the water control and field management, such as improving irrigation techniques and developing artificial rainfall. The major drawback however is that these methods can only be used in limited conditions or locations. By contrast, several recent studies have reported that a subset of beneficial bacteria -called plant growth-promoting rhizobacteria (PGPR) help plants to induce drought tolerance. PGPR are non-pathogenic, largely belonged to the gram-positive genera *Bacillus* and *Paenibacillus*. or gram-negative genus *Pseudomonas* spp. bacteria. As a group, PGPR which are known to enhance plant growth and development in both non-

stressed and stressed conditions by direct and indirect mechanisms (Glick *et al.* 2007, Nadeem *et al*. 2010, Zahir *et al.* 2004).

The direct mechanisms describe PGPR as bio-fertilizers, by producing organic compounds to promote plant growth or increase uptake of soil nutrients, whereas indirect mechanisms refer to 'PGPR -dependent biocontrol, including the production of antibiotics, Fe chelators (called also as siderophores), and external cell wall degrading enzymes (e.g., chitinase and glucanase) that perhaps hydrolyze the pathogen (i.e., fungus) cell wall (Zahir *et al*. 2004, van Loon 2007, Glick *et al*. 2007, Berg 2009, Hayat *et al*. 2010). In addition, selective PGPR strains produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase that triggers induced systemic tolerance (IST) of plants towards various abiotic stresses including drought (Yang and Kloepper 2008, Lim and Kim 2013), indicating that PGPR can prime plant drought tolerance.

1. Current Update on Plant Drought Resistance Pathways. Drought is a general term that describes a sustained period of significantly subnormal water or soil moisture supply (Viets Jr. 1971). Once plants perceive water deficiency, they initiate a series of physiological, cellular and molecular rearrangements that can result in drought stress tolerance (Shinozaki 2006).

1A. ABA-dependent drought resistance mechanism. In plants, drought prompts the pro-duction of abscisic acid (ABA), a major hormone in balancing the fitness (Denancé 2013). ABA modulates the expression of numerous genes and controls various intrinsic processes in plant growth and survival, including the inhibition of germination, the maintenance of seed dormancy, and the control of stomatal closure. During drought stress, ABA employs ABA-res- ponsive element (ABRE) binding protein/ABRE binding factor (AREB/ABF) transcription factors (TFs)

to regulate expression of many target genes. These TFs bind ABA-responsive element (ABRE) in the promoter region of ABA-responsive genes (ARGs), which is a con- served, 8-base pair-long *cis*-element (PyACGTGG/TC) with a core ACGT sequence (Naka- shima *et al*. 2009, Fujita *et al.* 2011). AREB has also been reported as a key positive regulator of ABA signaling pathway in plants. Indeed, transgenic plants overexpressing *AREB1/ABF2*, *AREB2/ABF4* or *ABF3* TFs clearly exhibited enhanced drought tolerance and increased ABA sensitivity (Lindemose *et al*. 2013).

WRKY TFs are another key regulator involved in ABA signaling. The WRKY group is one of the largest TF families involved in both repression and activation of various plant processes including abiotic and biotic stress responses (Rushion and Somssich 2010, Ulker and Somssich 2004, Rushton *et al*. 2010). Up-regulation of *WRKY* TF transcripts are largely correlated with elevated ABA signaling cascades and plant stomata closure that prevents the transpiration of water loss (Mansfield and Atkinson 1990). Furthermore, severak groups have reported in Arabidopsis that *WRKY70* appears to balance signaling branches between salicylic acid (SA) and jasmonic acid (JA) (Li and Brader 2006). For instance, *WRKY70* could promote SA signaling, which fosters positive regulatory modes to ABA signaling, while suppressing JA-dependent responses that mutually antagonize SA and ABA signaling (Li and Brader 2006). Furthermore, *WRKY70* suggesting a complex network circuitry intertwining growth regulation pathway (ABA signaling) and stress defense responses (SA and JA signaling)

1B. ABA-independent drought resistance mechanism. A previous study (Shinozaki and Yamaguchi 2000) demonstrated that a large number of genes are induced by water deficit but are not affected by exogenous application of ABA,suggesting that transcriptional responses to water

deficiency are regulated by both ABA-dependent and ABA-independent signal transduction pathways. In ABA-independent signaling, dehydration-responsive element binding (DREB), no apical meristem (NAM), ATAF, and cup shaped cotyledon (CUC) proteins are key TFs that control numerous downstream drought responsive genes.

In Arabidopsis, low temperature induced 78 (*LTI78;* also noted as *RD28A*) is a unique gene that is induced by ABA (Wilhelm and Thomashow 1993), –and can also be induced in mutant plants defective in ABA-biosynthesis and -signaling in response to drought and cold stresses (Yamaguchi-Shinozaki and Shinozaki 2006), suggesting the presence of a layer of safeguards (redundancy) in plant survival mechanisms during drought stress. In line with this scenario, Arabidopsis encodes eight (DREB)2-type proteins, of which DREB2A and DREB- 2B are major TFs that function under dehydration and high-salinity stress conditions (Shino- zaki 2003). *In planta* screening in parallel to high-resolution microarray analyses using trans- genic plants overexpressing *DREB2A* substantiated that DREB2A-inducible genes play an important role in drought stress tolerance (Sakuma *et al*. 2006).

Recently, several studies have started to highlight the pivotal roles of several plant-spe- cific TFs such as NAC (NAM, ATAF and CUC; i.e., ANAC096 and ANAC016) that could induce plant drought tolerance (Xu *et al*. 2013, Sakuraba *et al*. 2015). In rice, overexpression of *NAC* TFs *(*e.g., *SNAC1, OsNAC6/SNAC2, OsNAC5;* or *OsNAC10*) also showed significant improvement of drought tolerance (Nakashima *et al*. 2014). Interestingly, *NAC* transcripts are also induced by jasmonates that antagonize ABA and SA signaling while optimizing transcriptional responses towards of abiotic stresses such as tissue injury and excess lights, and

biotic stresses such as necrotrophic microbial infections and insect attacks (Nakashima *et al*. 2012).

2. PGPR: Bio-stimuli that Primes Drought Tolerance in Plants. Rhizosphere is the nar- row zone of underground soil, specifically influenced by the root system (Dobbelaere *et al.* 2003). This specific area around plant roots is rich in amino acids and sugars that are exuded by the growing plant (Gray and Smith 2005). These nutrients provided a suitable environment for many species of bacteria to grow and conduct physiological activities which can lead to benefi- cial, deleterious, or neutral effects on plant growth (Dobbelaere 2003). The beneficial free-living soil bacteria colonizing roots are commonly referred as PGPR (Kloepper *et al*.1989).

2A. Potential and current use of PGPR in agriculture. In a number of controlled laboratory and greenhouse experiments, as well as field trials, several PGPR strains successfully demonstrated to stimulate plant growth and survival through either direct or indirect mechanisms. Direct mechanisms are exerted by *i*) various compounds (e.g., phytohormones such as ABA) that are synthesized by the bacterium, or *ii*) facilitating the uptake of certain nutrients from the environment (Bhardwaj 2014). For instance, some strains from *Azotobacter* spp., *Azospirillum spp.*, *Phosphobacter* spp. and *Rhizobacter* spp. enhance uptake of nitrogen in *Helianthus annuus* (sunflower), leading to increases in plant height, plant weight, stem diameter, and seed filling (Dhanasekar and Dhandapani 2012). *Colletotrichum gloeosporioides* could suppress the anthracnose pathogens on mango, which offered an improved yield attri- butes (Vivekananthan *et al*. 2004).

Indirect mechanisms of PGPR refer to their activities in reduction or prevention of the deleterious effects caused by plant pathogens through production of antagonistic substances or by induction of resistance t (Glick 1995). For instance, a number of *Bacillus* spp. showed strong antagonistic activity against microbial and insect pathogens such as *Curvularia.lunata* (Basha and Ulaganathan 2002). In addition, it has been widely accepted that PGPR are capable of inducing plant resistance to abiotic stress via eliciting defense responses, termed induced systemic resistance (ISR, Viswanathan and Samiyappan 2002). ISR is developed as PGPR prime or potentiate the expression of defense genes for subsequent encounter to stresses (Ahn et al 2002, De Meyer *et al*. 1999, Kim *et al*. 2004, Tjamos *et al*. 2005), indicating that PGPR-mediated ISR is a common feature.

2B. Priming stress resistance and adaptation mediated by PGPR. Infection of plants by necrotizing pathogens or colonization of plant roots with certain beneficial microbes causes the induction of a unique physiological state called 'priming' (Conrath and Beckers 2006). Priming is a process that many plants could develop an enhanced capacity for activating defense responses to secondary biotic and abiotic stress. Recently, system modeling of genes induced by drought, cold or high salinity, conclusively scrutinize that plants can prime against various forms of biotic and abiotic stresses (Bray 1997, Ingram and Bartels 1996, Thomashow 1999, Hasegawa *et al.* 2000, Fowler and Thomashow 2002, Pastori and Foyer 2002, Seki *et al*. 2002a and b, Gerold Beckers 2006). The primed state can often be developed by stresses themselves; plants can acclimate to minor and temporal stresses, which potentiate and condition the priming state which confer effective defense and tolerance responses towards the same but stronger and prolong stress at the second time when plants encounter. However, recent studies demonstrated

that priming can also be developed by treating plants with various natural and syn- thetic compounds, or by colonizing plant roots with beneficial micro-organisms (e.g., PGPR; Jakab *et al*. 2005, Beckers 2007). Especially, a recent study reporting the capability of PGPR to induce priming drought tolerance in Arabidopsis, a model plant system, has opened the new door to the field of molecular plant-microbe interactions to further delineate the mode of action of not only priming but also the molecular principles in plant response to environ- mental stresses including in particular drought.

3. Future Directions and Perspectives in Sustainable Food Production. Drought is a major constraint that limits the crop production in agriculture (Chaves and Oliveira 2004). We are looking for an innovative approach to assist genetic engineering or molecular breeding research to upgrade plants' own defense capacities and improve yield and survival. Until now, the studies of the mechanisms of drought tolerance by plants have found various expla- nations. For instance, the accumulation of secondary metabolites and defense compounds in plants was associated with enhanced resistance to biotic and/or abiotic stress (Conrath *et al.* 2002). This indicates that plants have the capacity for what can be described as "memory," also called priming (Crisp, 2016). According to Crisp (2016), a key regulatory step governing whether memories are formed or forgotten is the period of stress recovery. During this period, plants balance resources allocated to acclimation against the benefits of resetting and reallocation into growth and/or reproduction (Crisp, 2016). As most studies on costs and benefits of induced plant resistance to abiotic stress have focused on situations in which the defense is activated directly by the inducing agent, the possibile costs of priming are relatively fewer than direct defense (Hulten, 2006). In the recovery state, plant growth can be maximized under favorable conditions and also be susceptible to

severe or recurring stress. In primed state, plants were prepared for local acclimation to various environments and delay growth or development. All above indicates that primed plants displayed significantly higher levers of fitness.

Recently, a number of studies have shown that PGPR inoculation could also induce priming for drought tolerance (Vriet *et al*., 2015). However, these studies have been lab tests, and the future challenge is to assess the role of priming under field conditions. To do this, we need for genetic or physiological markers to quantify the state of priming (Marieke van Hulten 2005). Hence, investigating the function and mechanism of priming will also be an exciting challenge for future research. Thus, we are trying to find out which gene or genes are responsible for priming plant drought stress and to cultivate a new genotype that would have a heightened drought tolerance.

Results

***P. polymyxa* primes drought tolerance on Arabidopsis.** To validate and further understand at the molecular and biochemical levels if cohabitations of PGPR assist plants to better cope with drought stress, we initially aimed to set up the standardized system of plant- PGPR interaction, preferentially using Arabidopsis plants in order to take an advantage of their rich scientific resources. Hence we corroborated and optimized an experimental condition that can heighten the states of drought tolerance in Arabidopsis by a gram-positive bacterium, *P. polymyxa* (Fig. 1, left panel), the only known PGPR strain that induced drought tolerance and expressed defense genes in Arabidopsis (Timmusk and Wagner 1999). In this condition, Arabidopsis, grown under 12-hr light/12-hr dark by watering every 2 d, consistently displayed enhanced drought tolerance (>70 %) when *P. polymyxa* strain CR1was applied at 1 x 10^8 cfu/mL to soils twice, at 2 and 4 d prior to applying the drought stress (Fig. 1, right panel). These results indicated that the treatment of *P. polymyxa* conveys the rearrangement of molecular and biochemical states in plant cells (i.e. Arabidopsis), which leads to prime systemic resistance or tolerance toward environmental stresses such as, in this case, water deficiency.

PGPR-induced priming of plant drought tolerance is broad phenomenon. To evaluate the significance of *P. polymyxa*-Arabidopsis interaction, the potential effects of *P. polymyxa* were examined for soybean (*Glycine max* L.).As shown in Figure 2, the *P. polymyxa*-treated group of soybean plants clearly showed induced drought tolerance, suggesting that PGPR-induced drought tolerance is not a species-specific mechani- sm, but rather a general mechanism throughout economically valuable crops. It is also likely that strains of other PGPR species could

also prime drought tolerance. To screen, in a high-throughput manner, PGPR strains capable of priming drought tolerance, we have developed a unique and simple drought tolerance assay method using the 12-well-plate sys- tem and Arabidopsis (Fig. 3). Thus far, the several trials of this assay using several PGPR strains obtained from Dr. J. Kloepper's lab have confirmed that *P. polymyxa*-treated Arabi- dopsis seedlings sustain green color longer than wither nonbacterized controls or seedlings treated with *Pseudomonas psychro- tolerans.* These results validate the applicability and feasibility of our new screening method to further isolate PGPR stains, capable of effectively priming drought toler- ance. Also the results show that *P. psychrotolerans* can be used as a negative control strain for future analyses and studies. Moreover, the preliminary assays repeatedly indicated that *B. amyloliq- uenfaciens* confers the priming of drought tolerance as effectively,- if not better, than *P. poly- myxa*, indicating the potential benefits of PGPR in various environmental stresses including drought stress. Now comprehensive screening of PGPR strains for stress cues can be done using our new assay.

***P. polymyxa* inoculation differentially regulates a subset of stress-responsive genes.** One of our objectives was to employ a network modeling approach including RNA-sequencing and computational analysis to infer the global landscapes of dynamic changes that occur in plant cells during the cohabitation with *P. polymyxa*. Towards that, we utilized four reference genes: *Pathogenesis-related Protein 4* (*PR4*), *Vegetative Storage Protein 2* (*VSP2*), *Rab* (G-protein)-*related 18* (*Rab18*) and *Low Temperature Induced 78* (*LTI78*, also called *RD29A*) (Timmusk and Wagner 1999) to determine optimum time points for the preliminary RNA-sequencing analyses. Since the second inoculation of *P. polymyxa* was critical to maximize the priming of drought tolerance in Arabidopsis (Fig. 1), total RNAs were prepared from *P. polymyxa*-treated

Arabidopsis leaves every 6 h for 96 h-post-the primary (1^{o}) *P. polymyxa*-inoculation (hp1^{o} i) and were subjected to semi-quantitative (q)RT-PCR assays to profile the level expressions of reference transcripts (Fig. 4). Unexpectedly, transcripts of both putative *P. polymyxa*-responsive genes (PRGs; *PR4, VSP2,* and *RAB18*) and non-PRG (RD29A) were all upregulated rapidly (<6 h) upon *P. polymyxa*-treatments. However, the transcript changes were somewhat irresolute, since those same genes were also rhythmically expressed in a 24-h period in the control plants. This oscillation of gene accusations indicated that the transcript levels of selected reference genes were likely regulated by light, more precisely changes in a day-night cycle (or circadian clock). Circadian clock, driven by an endogenous biological clock, regulate many biochemical, physiological and behavioral processes in a wide variety of organisms (Dunlap 1999). In plants, circadian clock is indeed involved in regulating stomatal opening, light perception, and other metabolic processes such as respiration and photosynthesis. However, little is known about molecular mechanisms that signal and regulate circadian rhythms (Mstsushika and Makino 2000).

***PR4, VSP2*, and *RAB18* expressions oscillate under control of the circadian clock.** To test whether the circadian clock controls the expression of the reference genes (*PR4, VSP2* and *RAB18*), their transcript profiles were investigated under circadian clock, using a high-resolution real-time qRT-PCR (Fig. 5). Plants were maintained under 12-h light/12-h dark diurnal conditions, and total RNA was extracted form replicate samples of plants at defined time points. The reference genes were rhythmically expressed during a 24-h period, with the expression of *PR4* highest at night (11pm) while those of *VSP2* and *RAB18* peak at day (11am to 3pm), respectively. This induction could be from ~3-fold (*PR4*) to over 80-fold *(VSP2),* indicating that

these genes are strongly influenced by circadian clock, even their basal level of expression are very different depends on the time point the samples were collected.

***P. polymyxa* response is gated by the circadian clock.** To further define the potential effects of *P. polymyxa* on the expression profile of the reference genes, their mRNA levels were measured by a high-resolution real-time qRT-PCR in every 6 h over a 96 h-period (Fig. 6). The transcript levels of *PR4* and *RAB18* were rapidly (>6 hp1^{o}i) upregulated with peaks at 12 hp1^{o}i, but reduced to the basal levels in a 24-h period, which demonstrates that *P. polymyxa* response of *PR4* and *RAB18* expressions are still under the circadian regulatory mechanisms. Since these two genes are wildly accepted marker genes for jasmonate and ABA signaling, these phytohormones must be involved in priming of drought tolerance, but *VSP2*, another marker gene in jasmonate signaling was, unlike the earlier study (Timmusk and Wagner 1999), not responsive to *P. polymyxa*-treatment More interestingly, *RAB18* is proposed as a so-called "memory gene" in priming drought tolerance during pre-drought acclimation, characterized to be produced considerably higher transcript levels during one or more subsequent stresses relative to the initial stress (Ding *et al*. 2011). Therefore, *P. polymyxa*-treatment showed the modulation of key regulators in priming (also referred to as conditioning, hardening, and acclimation) against environmental changes such as drought stress. However, effects of *P. polymyxa*-treatment on transcript level inductions of *RAB18* (and *PR4*) were gradually reduced; later peaks of *RAB18* (and *PR4*) mRNA levels became lower than those of earlier (or initial) inductions In particular, the secondary *P. polymyxa* inoculation at 48 hp1^{o}i exhibited little if any effect on level expression of both *RAB18* (and *PR4*). Considering that the secondary *P. polymyxa* inoculation

was often critical to develop the priming of drought tolerance, these results indicated the presence of other sets of genes needed to fully develop the priming.

***P. polymyxa* induces a subset of ABA-responsive and drought-induced genes.** Our RT-PCR assays then uncovered that *P. polymyxa* significantly up-regulates *RD29A* (called as also *LTI78*) transcript (Fig. 7) that was previously reported as non-PIGs (Timmusk and Wag- ner 1999). The discrepancy between the two experiments might be caused by the circadian clock -dependent oscillation of basal level expression of *RD29A*. Although *RD29A* is not considered as a memory gene, it is a critical marker gene of the ABA signaling pathway, supporting a critical role of ABA in priming drought tolerance. Indeed, another major ABA maker gene, *RD29B* is also strongly up-regulated upon *P. polymyxa* inoculation (Fig. 8). Interestingly, *RD29B* is - like *RAB18* as well as *ABCG13* - considered as an ABA signaling memory gene (Ding *et al*. 2012, 2013). However, not all memory genes are up-regulated by inoculation with *P. polymyxa*.. Our gene expression profiles revealed that another memory gene, *LTI30*, (Ding *et al*. 2013) was not regulated by *P. polymyxa* (Fig. 8). Together, our studies proposed that genes involved in ABA signaling and associated memory genes are important molecular elements in the PGPR-induced priming of drought tolerance, but those are not sufficient enough and need more complex cellular processes and metabolic pathways perhaps including JA signaling transductions.

Material and methods

Plant growth conditions. *A. thaliana* wild type (WT) Columbia (Col-0) and *G. max* (soybean) were grown in a growth chamber with 12 h day cycle (100-120 μE/m^2/s) at 22 ºC and 25 ºC with 60 % to 80 % relative humidity.

PGPR inoculation. *P. polymyxa* strain *CR1* was obtained from Dr. Z., Chen's group in the Agriculture and Agri-Food Canada. *P. polymyxa* was grown on Luria-Bertani (LB) media, and infiltrated with a syringe into soil around 3- to 4-week-old Arabidopsis and soybean plants at 10^8 colony-forming units (cfu) mL^{-1} in 8 mL H_2O, respectively. Mock inoculation was performed using ddH_2O. On soybeans, the first inoculation was made just after sowing the seeds, and the second was made 7 days after planting. The soybeans plants were watered until 13 days after planting at which point watering ceased, and data were collected 5 days later.

PGPR-induced drought tolerance assays. Arabidopsis plants were grown for 3 weeks, and plants were watered with 8 ml of H_2O every two days. Plants were then inoculated twice with the *P. polymyxa* solutions as described above. Drought stress was then applied by stopping watering. Photographs of plant responses were taken in 10 days after drought stress. For soybean experiments, plants were grown in 230 mL pots and watered every two days with 25 ml of water.

PGPR screening assay for drought tolerance in Arabidopsis. Arabidopsis was planted and grown in the 12-well-plate for one week with watering every two days (1 mL/well). Plants were inoculated with PGPR inoculums (10^8 cfu mL^{-1}) twice; once directly to seeds, and the second

time to soil around 1-week-old seedlings. Control seedlings were treated with H_2O, and all watering was stopped at 1 week.

Quantitative RT-PCR Total leaf RNA was prepared by using TEIzol reagent and Dierct-zol RNA MiniPrep Plus kit according to the manufacturer's instructions. The quality of RNAs was assessed by agarose gel electrophoresis and NanoDrop (A_{260}/A_{280} > 1.8 and A_{260}/A_{230} >2.0). RT reactions were performed by using an oligo(dT) reverse primer and a reverse transcriptase (Superscript-II, Invitrogen). The cDNA were assessed by quantitative (q)PCR with house-keeping gene *GAPDH*. PCR system cycled 40 times by using gene-specific primer sets (Table 1). The annealing temperature for the primers are 55 ^{o}C. To determine the relative abundance of target transcripts, the average threshold cycle (i.e., Ct) was normalized to that of *GAPDH* as $2^{-\Delta Ct}$, where $-\Delta Ct = (Ct_{,gene} - Ct_{,GAPDH})$. (Schmittgen and Livak, 2008)

Semi-quantitative RT-PCR A total of 1 μL of cDNA prepared as described earlier was used for semi-quantitative RT-PCR, performed with Taq 2X Master Mix. The annealing tempera- ture for primer pairs was 53C and the PCR performed 40 cycles. Each cycle consisted for 95°C for 30 s, annealing temperature for 30 s, and 72°C for 30 s; the final step occurred at 72°C for 5 min.

Discussion

Over the last decades, global warming - increases in the Earth's surface temperature - has rapidly reduced water availability in agricultural farming areas and resulting in 'drought' as one of main environmental stresses that now causes severe losses in crop yields (Fa- rooq 2009). A number of reports have recognized the urgent needs of developing unique and sustainable management strategies such as new resistant cultivars towards drought stress in order to maintain, if not enhance, the yields of food and biofuel crops (Jones, 2004).However, finding such potentially drought tolerance varieties requires information not yet known on the stress physiology of plants exposed to drought,, due largely to our little knowledge on the stress physiology of plants during drought and the global warming. As an initial effort to isolate key molecular elements which heighten resistance and/or tolerance of plants against abiotic stresses in particular drought, we aimed to discern plant drought-defense mechanisms from drought-responsive metabolisms. We found in this study that *i*) pre-treatment of PGPR (i.e., *P. polymyxa*) primed drought tolerance in Arabidopsis and soybean plants, through *ii*) triggering signalling transductions of the defense hormones ABA and JA, *iii*) which in turn rapidly induced the expression of drought tolerance-associated transcripts such as *RAB18* and *RD29A*. Interestingly, *iv*) a subset of *P. polymyxa*-induced genes (e.g., *RAB18* and *RD29B*) are known as 'memory genes' whose transcript levels were considerably higher during one or more subsequent stresses relative to the initial stress (Ding *et al*. 2012), indicating that *v*) PGPR-treatments lead to the chromatin modification of defense (or memo- ry-) related genes in alerting and equipping the cellular states of plants against drought and other environmental stresses.

Our *in planta* and transcription analyses demonstrated that the PGPR-induced priming of drought tolerance is conditioned through transcription-level regulations (or PGPR-induced drought tolerance genes, PIRGs). On the other hand, a number of PGPR-responsive genes (PRGs) are directly or indirectly involved in plant growth promotion (Shaik, 2012). Thus PIRGs, a subset of PRGs, also function in assisting and coordinating the plant growth promotion, which are one of the most valuable traits of PIRGs since most, if not all , of drought-responsive genes (DRGs) cause the plant growth retardation (e.g., stomatal closure, Moon, 2016). These were a major pitfall to generate a useful generic resource from DRG database. The overexpression of DRGs, indeed, enables to enhance drought resistance or tolerance in the broad range of plant species (Moon, 2016). However, these overexpressions mostly render stomatal closure and prevent water evaporation, which in turn delay plant growth and development. Therefore, DRGs are generally not applicable for the development of drought and water management programs. By contrast, PIPGs belong to PRGs likely show little, if any, effect on the plant growth retardation. Thus, comprehensive identification of PIPGs will provide the unique and practical molecular repertoire in genetic engineering or molecular breeding approaches to upgrade a plant's own defense and growth capacities and improve yield and survival for food or biofuel crops.

In plants, priming refers to the heightened state of stress-tolerance or stress-resistance. Exposure to stresses conditions priming and enables plants to cope more effectively with subsequent stresses when they occur. For instance, prior occurrence of a minor and brief dehydration can prime drought tolerance towards major and extended drought stresses. However, treatment of pre-dehydration can be technically challenging for the field application. Thus, the

main research focus of our study was targeted to identify - so-called - ‘memory genes (i.e., *RAB18* and *RD29B*) that associated with drought tolerance or resistance, and ABA sig- naling (a main drought resistant-related phytohormone; see Literature Review) (Ding *et al*. 2012). Interestingly, our study has revealed, for the first time, that pre-PGPR-treatment is capable of inducing the expression of *RAB18* and *RD29B* without actually encountering the pre-dehydration, proposing that PGPR-treatment can be the viable alternative to pre-dehydration for the field application in inducing drought tolerance or resistance.

Plant memory is often expressed as heightened molecular response that occurs after exposure to a subsequent stress and plant memory is also known as an enhanced or more rapid response (Crisp *et al*. 2016). One mechanism of memory genes may be the variable expression level of important signaling metabolites or transcription factors which explained how plants adjust their metabo- lism when they are exposed to biotic or abiotic stress (Crisp *et al*. 2016). For instance, Arabi- dopsis pre-trained by dehydration stress could survive longer than non-trained plants by altered drought related genes’(*e.g. RD29B, RAB18*) responses after an initial dehydration stress (Ding, 2012)

Until recently, reported cases of durable stress ‘memory’ in plants mostly involved transposon activation, homologous recombination, DNA methylation or small noncoding RNAs which offer another possibility of the mechanism is related to chromatin states. The main players of plant stress responses at the chromatin level include DNA methylation and demethylation enzymes, histone modification enzymes, histone variants, chromatin remodeling complexes and other chromatin associated factors (Vriet *et al*., 2015). The model of chromatin-level regulation

of plant defense response was described as 4 states. Under optimal growth conditions, a stress-responsive gene is kept in a repressed (silenced) state. After exposed to short stress, the deposition of chromatin activating marks and/or the removal of repressive marks establish a sensitized state. Genes in sensitized state maybe weakly transcribed or continue to be inactive until the long stress duration or high intensity induces an activated state. When the stress stops, genes "calm down" to the sensitized state again. As long as the repressed state has not been reestablished, a second moderate or brief stress is sufficient to reactivate the gene and lead to hyperactivated state, in which genes have shown a further increase in the rate of transcription (Vriet *et al*., 2015).

PIPGs also include non-memory, but ABA-dependent, drought tolerance genes (i.e. *RD29A*), and JA (a major plant stress assimilation hormone)-associated PR genes (e.g. *PR4*, Fig 4 and 5), indicating that PGPR rearrange the pattern of global gene expressions and metabolic path- ways in programing plants' optimal phenotypes under different ecological condition. Hence, further investigation employing the network modeling approach will help delineate the global landscapes of dynamic changes that occur across multiple levels in the PGPR-mediated sig-naling network. Our study revealed the model trajectory of PIPG and PRG expressions, that will allow us to determine time courses for the genome sequencing analysis (i.e. RNA sequencing; our next aims). We will acquire spatiotemporal PGPR transcriptomic landscapes in various biological/genotypic conditions that will be subsequently subjected to pathway analysis, gene set enrichment analysis, generation of co-expression network and dynamic regulatory analysis. Collectively, our network-centered analyses will generate interactome models toinfer and assess

biological functions of PRGs, understand biological processes and molecular pathways, and predict and prioritize informative candidate genes for further investigation.

Table

Table 1. Oligonucleotides used for RT-PCR.

Genes	Directions	Sequences (5′-3′)
LTI30 At3g50970	Forward	*GGG ACT AAC ACG GCT TAT GG*
	Reverse	*CCT GGC AGT TGC TCT TTA AT*
ABCG13 At1g54160	Forward	*CCG TGC TAA ACT CGA AGC TC*
	Reverse	*AGC ATG AAG ATG GCG AGA CT*
RD29B At5g52300	Forward	*ACG AGC AAG ACC CAG AAG TT*
	Reverse	*AGG AAC AAT CTC CTC CGA TG*
RAB18 At1g43890	Forward	*TAG CTC GGA GGA TGA TGG*
	Reverse	*CAT ATC CGG ATC CCA TGC C*
LTI78 (RD29A) At5g52310	Forward	*ATC GAT GCA CCA GGC GTA A*
	Reverse	*TGC ATC GTG TCC GTA AGA GG*
VSP2 At5g24770	Forward	*GGA TAC GGA ACA GAG AAG AC*
	Reverse	*AAC TTC CAA CGG TCA CTG AG*
HEL At3g04720	Forward	*GCA AGT GTT TAA GGG TGA AGA*
	Reverse	*TAG CCA AAA CCA TCG GTG TC*
GAPDH At1g13440	Forward	*TTG GTG ACA ACA GGT CAA GCA*
	Reverse	*AAA CTT GTC GCT CAA TGC AAT C*

Figures

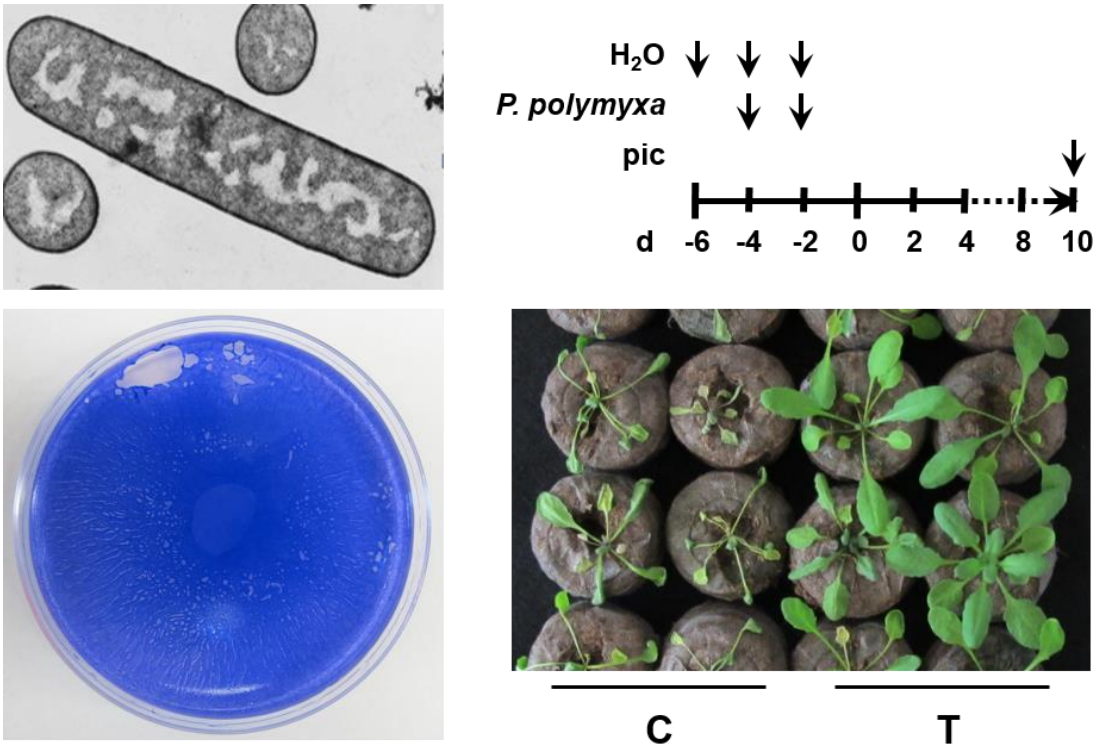

Figure 1. Gram-positive PGPR, *P. polymyxa*, is capable of priming drought tolerance in Arabidopsis plants. All plants were grown for 3 weeks while watering in every 2 days (8 mL). Then, two inoculums of *P. polymyxa* with 10^8 cfu mL^{-1} (8 mL) were soil-applied around plants at 2 and 4 day in prior to stopping watering (drought stress). Control group was treated with H_2O, and the representative photograph was taken 10 day-post drought stress. C = control, T = treated.

Figure 2. *P. polymyxa* is capable of priming drought tolerance in *G. max* (soybean). Soybean seeds were treated with 10^8 cfu mL^{-1} of *P. polymyxa* for 30 min, and planted on the 2.5' pots. Plants were then grown for 3 weeks with watering 12 mL in every 2 days. The representative photograph was taken 14 day-post application of drought stress.

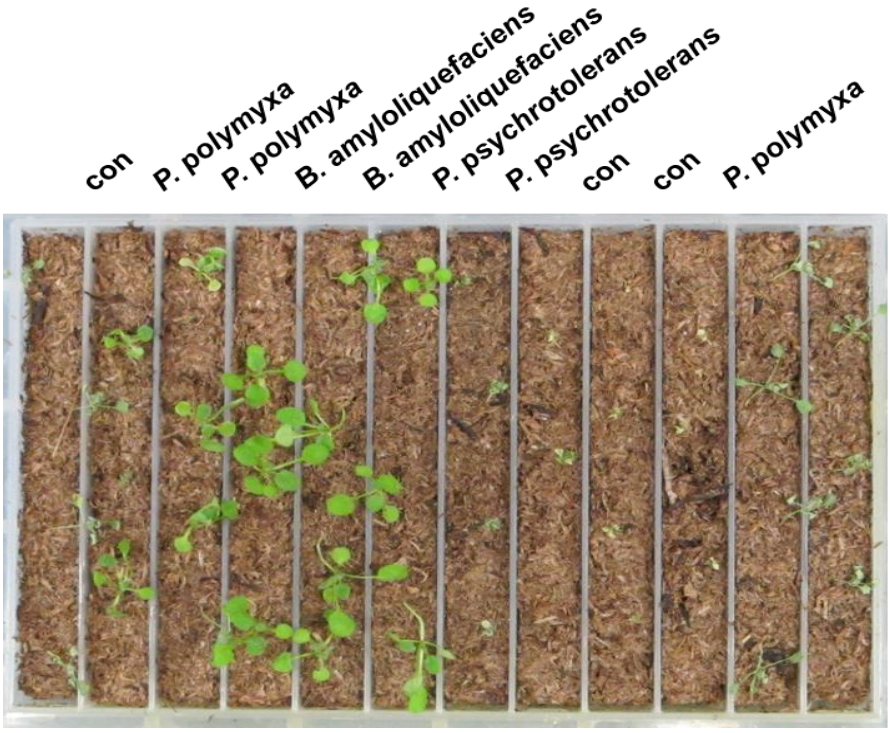

Figure 3. High-throughput approach to screen PGPR strains, capable of priming drought tolerances. Arabidopsis seedlings were grown in the 12-well-plate for 1 week while watering in every 2 days (1 mL). Plants were inoculated with three different PGPR inoculums (10^8 cfu mL^{-1}, *P. polymyxa*, *B. amyloliquenfaciencs* or *P. psychrotolerans*) twice; once directly to seeds, and the second time to soil around 1-week-old seedlings. Control seedlings (con) were treated with H_2O, and all watering was stopped at 1 week. Representative photograph was taken 4 day-post application of drought stress.

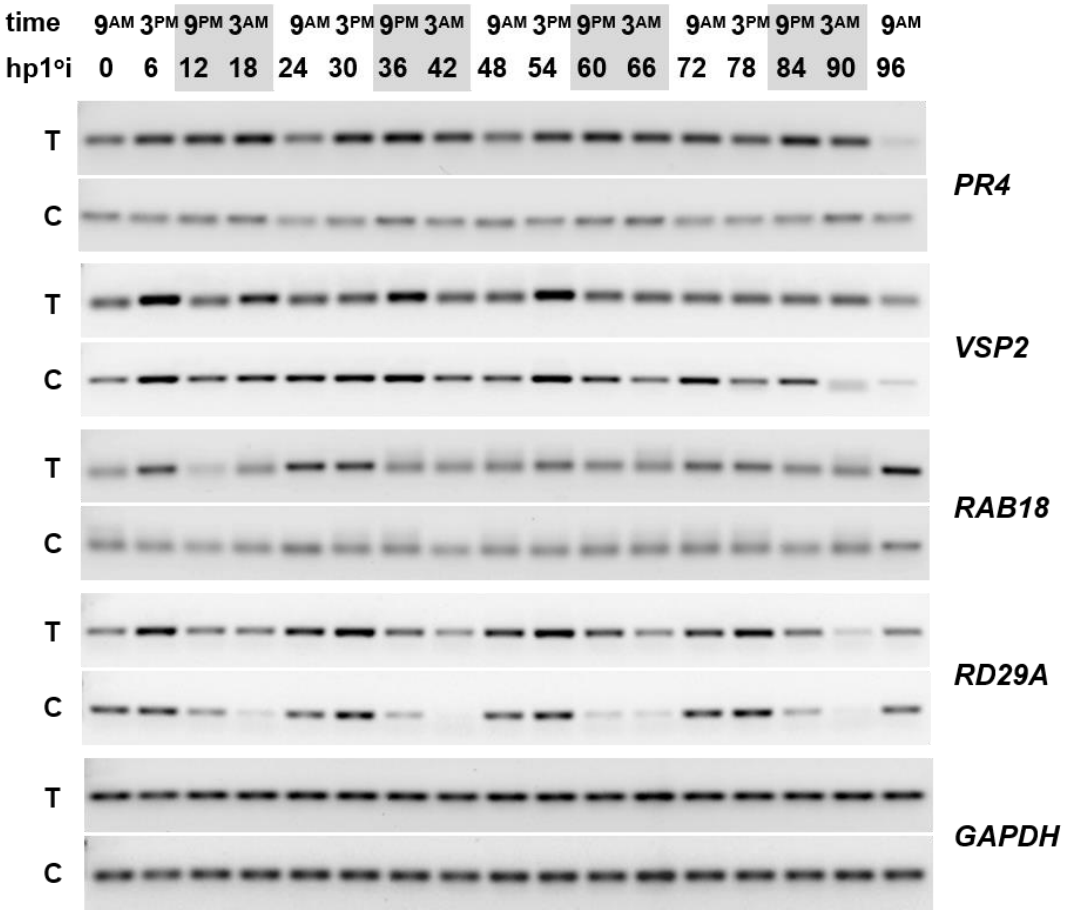

Figure 4. Time-resolved rearrangement of putative *P. polymyxa*-responsive gene expressions. Semi-quantitative RT-PCR of *PR4*, *VSP2*, *RAB18* and *RD29A* (*LTI78*) transcripts (Timmusk and Wagner 1999) in Arabidopsis (wild type, ecotype Columbia-0), grown under 12-hour light/12-hour dark conditions for 3 week. Total RNAs were prepared from leaves in every 6 hour-post-the primary *P. polymyxa* inoculation (hp1°i, 9-am) for 96 hp1°i. Note that inoculum concentration of *P. polymyxa* was 10^8 cfu mL^{-1}, and the 2nd inoculation was carried out at 48 hp1°i. Expression levels of *GAPDH* were used as a loading control (means ± SD; $n = 3$). C = control, T = treated. Grey shades in times indicate night periods.

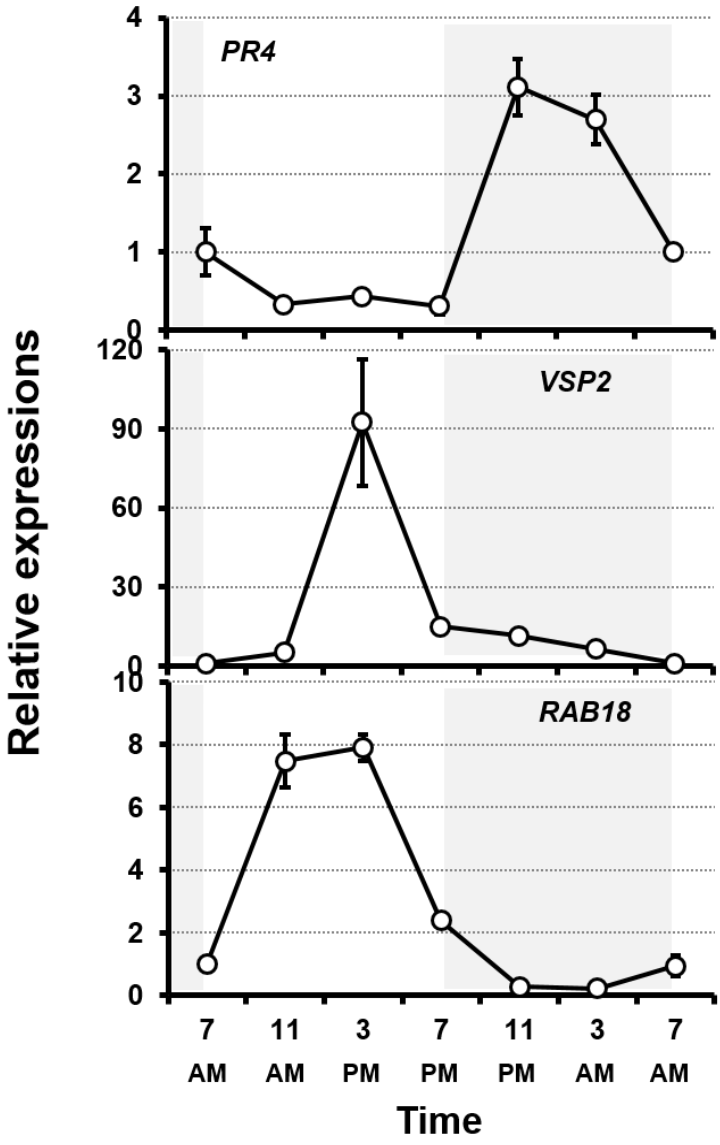

Figure 5. Transcript levels of putative *P. polymyxa*-responsive genes are regulated by the Circadian clock. High-resolution real-time quantitative (q)RT-PCR of *PR4*, *VSP2* and *RAB18* in Arabidopsis plants, grown under 12-hour light/12-hour dark conditions for 3 week. Total RNAs were prepared from leaves in every 4 hour starting from 7 am in the morning as noted. Values were normalized to the expression of *GAPDH* (means ± SD; $n = 3$). Grey backgrounds indicate night periods.

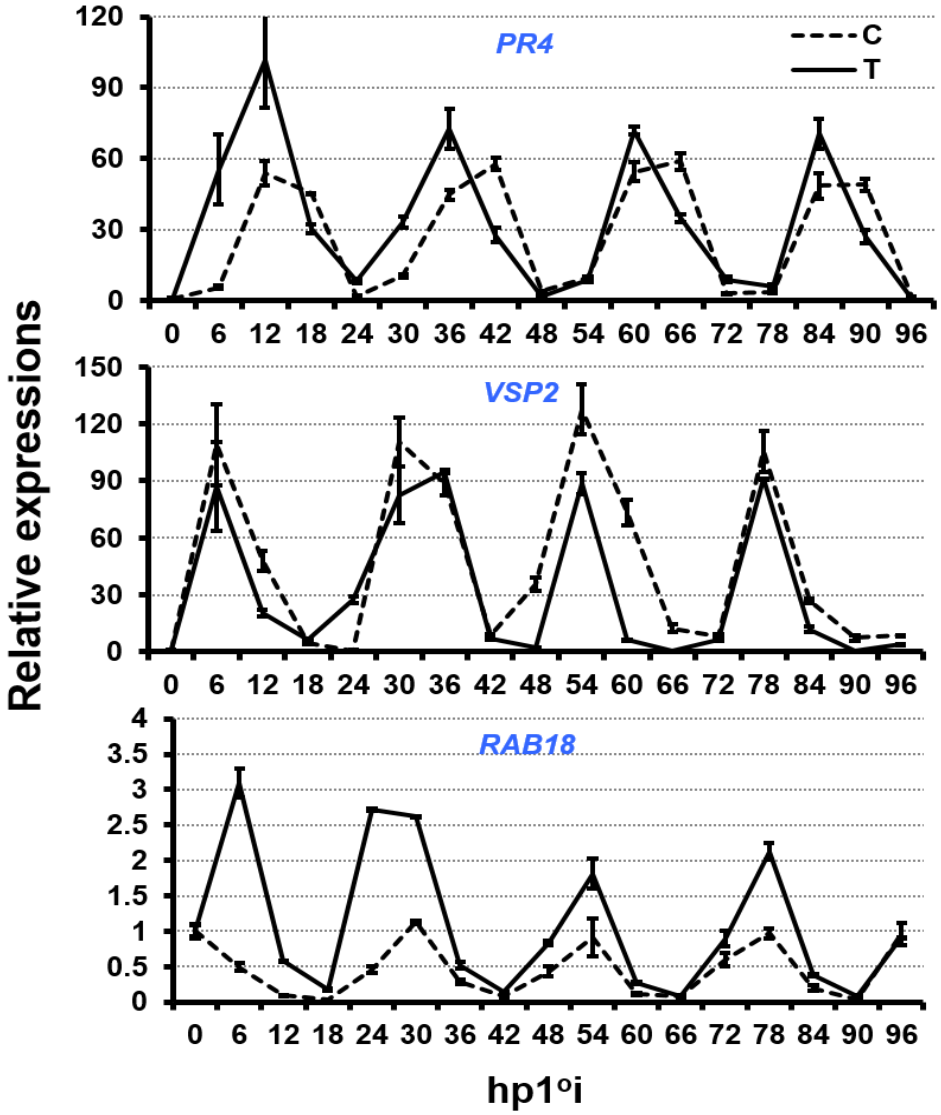

Figure 6. Circadian clock-dependent, *P. polymyxa*-responsive regulations of *PR4*, *VSP2* and *RAB18* transcripts. Time-resolved real-time qRT-PCR of putative *P. polymyxa*-responsive genes in Arabidopsis plants, grown under 12-hour light/12-hour dark conditions for 3 week, following the inoculation of *P. polymyxa* (10^8 cfu mL^{-1}). Total RNAs were prepared from leaves in every 6 hp1ºi (9 am) for 96 hours. Note that the 2 nd inoculation of *P. polymyxa* was carried out at 48 hp1ºi, and values were normalized to the expression of *GAPDH* (means ± SD; *n* = 3). C = control, T = treated.

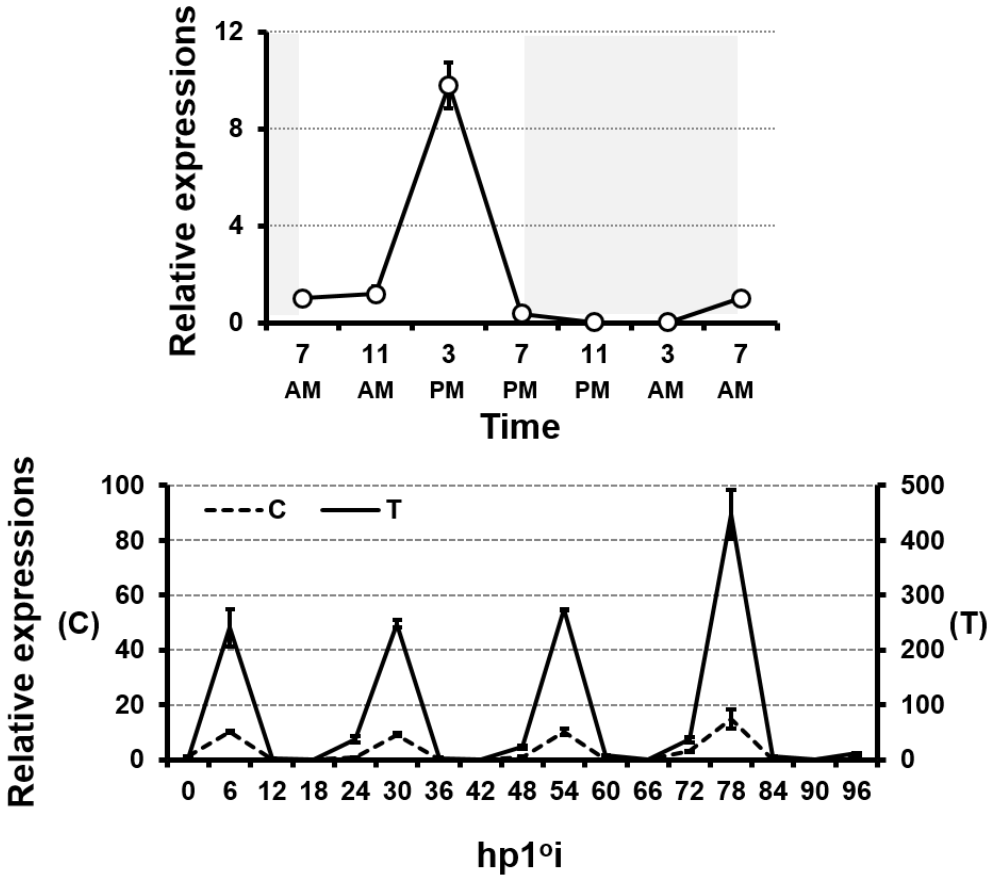

Figure 7. Circadian clock-dependent, *P. polymyxa*-responsive regulations of *RD29A* (also called *LTI78*) mRNA. Methods for PGPR inoculations, RNA preparations and qRT-PCR were described in the legend of Fig. 6.

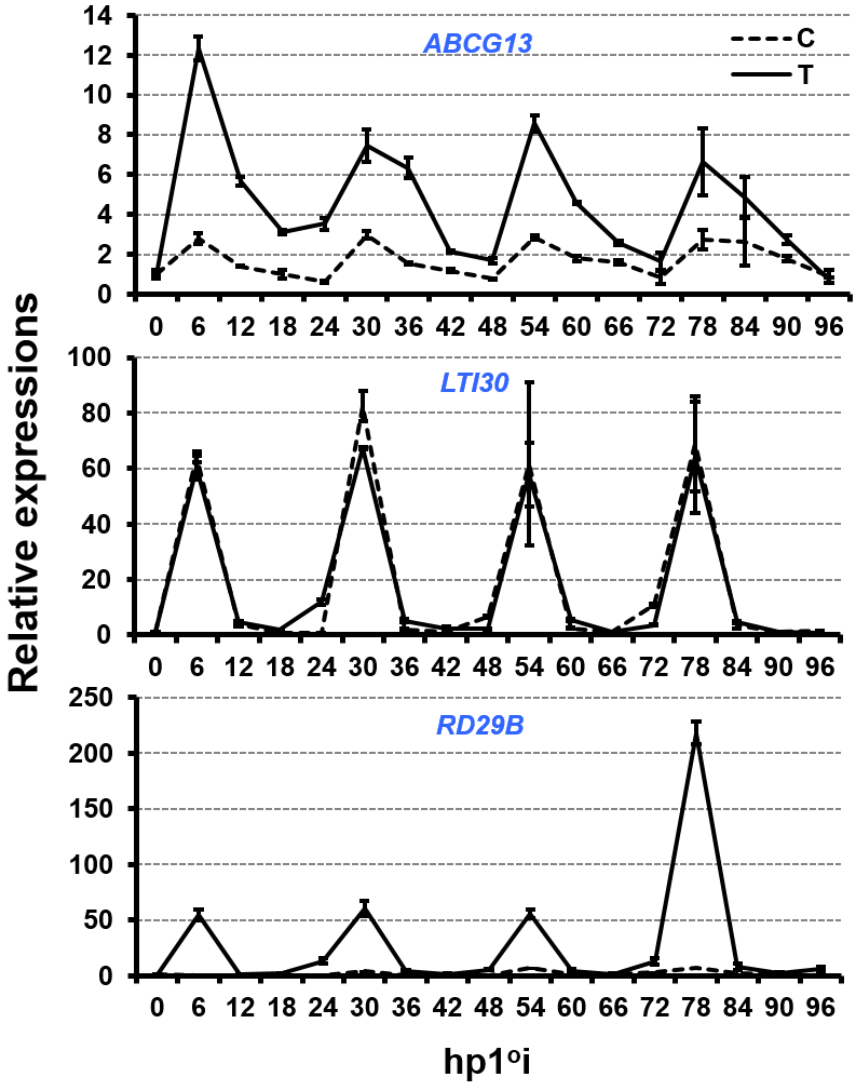

Figure 8. Circadian clock-dependent, *P. polymyxa*-responsive regulations of the memory genes of plant drought tolerance (*ABCG 13*, *LTI30* and *RAB18*; Ding *et al*. 2012). Methods for PGPR inocu-lations, RNA preparations and qRT-PCR were described in the legend of Fig. 6.

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