Investigating the molecular mechanism and genetic utility of plant growth-promoting rhizobacteria-mediated induced systemic tolerance

by

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Abstract

Drought is a major abiotic stress limiting agriculture production. Most research is focused on identifying and subsequently overexpressing plant drought-responsive genes (DRGs) to enhance the drought tolerance of plants. However, such research has shown undesired phenotypes on plants as a side effect. Recently, two DRGs *(RD)29A* and *RD29B* were identified to be induced by *Paenibacillus polymyxa* CR1, a plant growth-promoting rhizobacterium capable of priming drought tolerance (i.e., Induced Systemic Drought Tolerance, IST) and concurrently stimulating plant growth, play pivotal roles in defense responses against drought. This study was conducted in order to replicate the IST molecular mechanism of PGPR *P. polymyxa* CR1 by overexpressing *RD29* genes, aimed to circumvent the growth trade-off typically associated with drought tolerance in plants using strong circadian COR15A promoter. Compared to strong constitutive 35S promoter, native RD29 circadian promoters, strong circadian COR15A promoter showed normal growth in optimal conditions. These results hold promise for generating economically viable drought-tolerant crops. Similarly, during drought stresses, antioxidant enzymes such as Glutaredoxin (GRXs) are produced to maintain the cellular redox homeostasis in plants and generally divided in 3 classes in Arabidopsis model plants. These are a heat-stable oxidoreductases that use reduced glutathione (GSH) to metabolize thiol-disulfide exchanges with protein-disulfides, namely 'deglutathionylation'. Specially, the class III GRXs functions in various hormone signaling to coordinate and survival under environmental stresses. For instance, GRX480 is induced by jasmonates and salicylic acid, and in turn binds/controls TGA transcription factors (TFs), which actuates general defense mechanisms. However, the biochemical activity and mode of GRX480 have not been characterized due to its C-terminus hydrophobic tail that hinders the expression and purification of recombinant proteins. In

this study, we employed a maltose-binding protein (MBP) as a solubility enhancer, and successfully prepared a recombinant GRX480. Using the plasmid pKDL66, an amino (N)-terminus HIS-tagged MBP was fused to the N-terminus of GRX480 and induced under the control of T7 promoter in Escherichia coli BL21 (DE3) cells. The HIS-MBP tag was later removed by tobacco etch virus proteases, and the nickel affinity column chromatography. GRX480 confirmed its oxidoreductase activity, reducing 0.39 μM of S-glutathionylted bovine serum albumin per min ratio. Its apparent Km values were determined as 6.4 μM, lower than known Km values of class I GRXs (> 80 μM), suggesting that increased GSH levels induced by jasmonate and salicylic acid signaling could re-arrange GRX480 and TGA interactions, and TGA-dependent transcriptions.

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Chapter 1

Introduction

Plants are exposed to a range of different environmental stresses. Among these, drought is considered one of the most severe and costliest abiotic constraints on the crop production, as its annual losses totaled ~$ 30 billion worldwide [1]. Lately, global climate changes further limited water resources and aggravate drought conditions. By 2050, it is predicted that about one-third of global soils will suffer from drought [2]. To curb the drought, a common approach widely, currently used is irrigation [3]. However, these approaches more and more unreliable and costly [4]. Thus, there is a pressing need to develop sustainable and innovative approaches, not only addressing immediate needs to alleviate drought by replenishing moisture to the soil, but also generating new cultivars with improved drought resilience. Successful development of drought-tolerant crops will reduce our dependence on irrigation and contribute to the broader sustainability of agricultural practices.

In response to drought and excess salt and temperature, plants often react to avoid and/or endure them by closing stomata, decreasing photosynthesis and leaf growth/canopy, and increasing reactive oxygen scavenging activity and root length [5]. In addition, plants also produce chemical and signaling substances like phytoalexins, reactive oxygen species (ROS) and phytohormones [6], which orchestrate a layer of defense machineries through coordinating differential gene expressions, fostering plants more resilient and growing well under stress conditions [7, 8]. For instance, defense hormones such as jasmonates, salicylic acid (SA), ethylene (ET) and abscisic acid (ABA) interact/crosstalk with growth hormones like auxins, cytokinin and gibberellins; together control the activation of defense responses to various environmental stresses [9, 10]. Especially, ABA regulates a range of signaling and metabolic pathways that assist plants to mitigate drought and climate challenges [11, 12]. It carries out two main tasks,

controlling its own metabolisms and transports, as well as interacting with core transcription factors (TFs) in stress responsive signaling. These are essential signaling elements, crucial for feedback, maintaining hormone concentrations in tissues and facilitating plants' healthy growth and development [6, 11].

Plant defense responses against drought stresses

Plants facing drought stress convey a series of morphological (e.g., decrease leaf area and numbers, increase root length and leaf aging, induce early maturation, and change growth stages), physiological (e.g., stomata closure, photosynthesis, osmotic balance, and water transpiration and transmission), and biochemical changes (e.g., antioxidant, chlorophyll, proline, hormone, secondary metabolite contents), which enable plants to survive, termed as drought resistance. However, such intrinsic resistance strategies appear not enough for plants to effectively combat different drought circumstances. Thus, large efforts have been invested in utilizing drought-responsive genes (DRGs) in order to develop tolerant or resistant crop cultivars and understand plant cellular mechanisms, which might provide the key to improving plant drought tolerance.

1. *ABA-Dependent and Independent Drought Resistance Mechanism.*

ABA is a key hormone fostering plants' drought responses, by running a variety of innate cellular processes, including the regulation of stomatal closure, the prevention of germination, and the preservation of seed dormancy [13]. In response to drought, plants differentially express ABA-dependent and -independent DRGs [14]. For instance, Arabidopsis includes three *ABA-Responsive Element (AREB)*-*Binding Proteins* (*AREBs*) or *ABRE-binding factors* (*ABFs*) such as *AREB1*/*ABF2*, *AREB2*/*ABF4* and *ABF3*, which play

positive roles in ABA signaling, according to their overexpression studies [15-21]. WRKY TFs are another key regulator involved in ABA signaling. WRKY inductions are correlated with elevated ABA signaling and stomata closure, which prevents water transpirations involved in both repression and activation of various plant processes under drought [22-26]. The upregulation of WRKY TFs is largely correlated with elevated ABA signaling cascades and stomata closure, which prevents water transpirations [25]. On the other hand, the same study [25] demonstrated that a large number of genes, not affected by the exogenous application of ABA, are induced by water deficit, suggesting that DRGs are regulated by both ABA-dependent and ABA-independent signaling pathways. The latter employs *Drought-Responsive Cis-Element Binding Protein* (*DREB*) TFs that coordinates transcriptions under drought and salt stress conditions [27, 28]. Recently, several studies have started to highlight the pivotal roles of *NAC* (*No Apical Meristem (NAM), Arabidopsis Activation Factor (ATAF) and Cup-shaped Cotyledon (CUC)*) TFs in the activation of plant drought tolerance [29, 30]. In rice, the overexpression of *NAC* (e.g., *SNAC1, OsNAC6/SNAC2, OsNAC5 or OsNAC10* showed the significant improvement of drought tolerance [31]. Interestingly, NAC TFs are also induced by jasmonates that antagonize ABA and SA signaling while optimizing transcriptional responses towards other abiotic stresses such as ROS, tissue injury and excess lights, and biotic stresses such as necrotrophic microbial infections and insect attacks [32]. As a result, genes involved in ABA signaling play a key role in the production of transgenic plants that could enhance drought tolerance.

Current Progress in Biotechnology toward Plant Drought Tolerance

Thus far, major research has revolved around leveraging genetic traits that improve plants' responses to drought via conventional and genetic breeding. Toward that, a large number of studies have isolated/characterized the induction of gene

expressions triggered by drought in a model plant system Arabidopsis plant [33, 34]. These studies have influenced the trajectory of drought-related studies and led to the identification of "candidate genes" likely to confer drought resistance [35]. Indeed, many DRGs have been identified as candidate genes and tested in various crop species (Table 1). These DRGs are often divided into two categories; one involved in cellular defense such as osmoprotectant, membrane stability and transport, and cellular detoxification. The other contains TFs and signaling molecules [36]. Osmoprotectants like glycine betaine, mannitol and trehalose are one of the favorite targets for genetically modified crops, to enhance tolerance by protecting important macromolecules. Increased accumulations of these compounds by transferring genes in transgenic wheats indeed display the significant improvement of drought tolerance [37]. Drought tolerance can also be achieved by osmotic adjustment and chaperone-like activity in stabilizing cellular membrane and proteins, as well as the detoxification of scavenging ROS [38]. Plants also promote the expression of numerous TFs, which then factor the up or downregulation of a series of DRGs by binding to the enhancer or promoter region of genes [39-43], coordinating increased tolerance to abiotic stressors by their overexpression's [44-46]. However, a majority of those transgenic plants triggers stomatal closure and reduction, resulting in limited respiration under drought stress, but in turn accompany slowed growth and senescence [13, 47-49]. 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 are very costly and required special technical knowledge for successful operations.

Potential Use of Plant Growth-Promoting Rhizobacteria in Agriculture

Recent studies have demonstrated the versatile ability of plant growth-promoting rhizobacteria (PGPR) in enhancing both overall plant growth and drought tolerance [50]. These beneficial bacteria colonize plant roots and stimulate plant growth and survival through either direct or indirect mechanism. A direct mechanism involves nitrogen fixation, phosphate solubilization, micronutrient enhancement, and the production of enzymes 1-Aminocyclopropane-1-carboxylate deaminase (ACC) and plant growth hormones such as Indole -3 -Acetic Acid (IAA), auxin, cytokinin, gibberellins, ethylene and 2, 3-butanediol [51, 52]. For instance, *Bacillus* M3 significantly increases the total nutrient content (N, P, K, Ca, Mg, Fe, Mn, and Zn) in plants. On the other hand, other PGPR assist plant health by suppressing pathogens through the production of antibiotics or siderophores, thereby mitigating the adverse effects of diseases on plants and indirectly promoting growth and yield. Plants inoculated with *P. aeruginosa* strains under drought stress exhibited ~300% yield increments compared to control stressed plants [53]. Along with drought, PGPR has been reported to confer other abiotic stress, including salt and heavy-metal stresses in plants [4].

1. *PGPR Prime Drought Tolerance in Plants*

PGPR inoculations enhance drought tolerance [54]. During water deficiency, PGPR could improve root and shoot morphology, antioxidant and secondary metabolite productions, biofilm formations to increase plants' water accessibility [55-58]. For instance, the inoculation of *Azospirillum* spp. are able to induce IAA signaling and boost lateral root formations and growth, improving water and nutrient uptakes in wheat [59]. *P. aeruginosa* also can help increasing root and shoot length, and upregulated abiotic stress genes, together conferring drought tolerance in mung beans [60] . In addition, a number of studies explains it as Induce Systemic Tolerance (IST, also called priming), which is a heightened defense capacity against abiotic stresses [61]. 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 synthetic compounds, or by colonizing plant roots with beneficial microorganisms (e.g., PGPR) [62, 63]. However, the mechanism of priming is still remain largely unknown. Hence, investigating the function and mechanism of priming will also be an exciting challenge for future research.

2. *Induced Systemic Tolerance (IST)*

Lately, increased attention has been drawn to the possible efficacy of bio-stimulants in crop production, especially PGPR. In the soil, thousands of PGPR strains inhabit plant root systems, forming a complex eco-community that stimulates plant growth and immunity against microbial pathogens, pests, and insect herbivore insects. PGPR also can elicit **IST**; a state of heightened defenses activated throughout the plant, providing enhanced tolerance to various <u>abiotic</u> constraints including drought, salinity, and extreme temperatures [9-12]. In this context, our recent study has demonstrated that a common soil bacterium, *P. polymyxa* CR1, induces a unique subset of defense gene expressions and develops IST against drought while concurrently promoting growth and development in tomato, Arabidopsis, and soybean plants [5]. One caveat is that PGPR often displays little reproducibility in the field, because many endo/exogenous factors cause them to be unable to colonize plant roots [13]. Hence, a more sustainable approach is, instead of the direct application of PGPR, to transform plants with PGPR-responsive genes (PRGs) and their mechanisms. These can then be used to prime IST and generate drought-tolerant crops via well-established genetic engineering methodologies. Though molecular components and the mechanisms that underlie IST development and

plant/PGPR interactions are still largely elusive [11], their exploration is one of the goals of this proposal.

It is worth noting that earlier studies proposed that IST against drought occurred due to PGPR induction of growth hormones (e.g., auxin and cytokinin), which promoted root growth that resulted in an increased ability to obtain water during drought [12, 33]. However, we recently showed that more than two dozen PGPR isolates could not enhance drought tolerance in plants despite being able to stimulate better root growth than *P. polymyxa* CR1 [5], indicating that the modes of IST are not completely explained by PGPR-stimulated root growth and development.

3. *Responsive to Desiccation (RD)29s are key PGPR-Responsive Genes (PRGs) in IST Activations*

As an initial step to delineate cellular mechanisms that activate IST in plants, we searched PGPR-responsive defense genes in extracts of Arabidopsis via qRT-PCR. From this, we discerned that *P. polymyxa* CR1 induces the expression of two DRGs, *RD29A* and *RD29B*. Importantly, their expressions were downregulated when exposed to negative control PGPR such as *B. velezensis* and *P. oryzihabitans*, incapable of priming drought tolerance [5]. In line with these findings, the T-DNA KO insertion mutation of *RD29A* or *RD29B* impaired IST and showed increased susceptibility to drought. In addition, the mutant plants showed little difference in growth in optimal conditions, compared to WT. These results show that *RD29A* and *RD29B* ***i***) convey plant basal and systemic defenses priming against drought, while promoting growth fitness under optimal conditions, ***ii***) could aid in deciphering the mechanism of IST and ***iii***) serve as key transgenes in upgrading plant survival capacity under both drought and well-watered conditions.

4. *Characterize the Circadian Clock Regulation of RD29s in IST*

Recent findings have identified *RD29*s as circadian clock genes crucial for priming IST by PGPR. The circadian clock, influenced by the earth's rotation, empowers plants to adapt to daily environmental fluctuations, orchestrating the timing of energy production and consumption. Beyond its role in daily rhythmic processes, the circadian system regulates responses to both biotic and abiotic stresses. In addition, the circadian system plays a role in regulating responses to both biotic and abiotic stresses. Global transcriptomic analyses conducted in Arabidopsis reveled that almost half percentage abiotic stress responsive genes were under the control of the circadian clock gene [64].

The promoter region of *RD29*s contains one or more DRE (Dehydration Response Element) motifs in their promoter regions gated by two central circadian clock oscillators, *Late Elongated Hypocotyl (LHY)* and *Circadian Clock Associated 1 (CCA1)* [33, 65-67]. *LHY/CCA1* are morning expressing TFs, binding and activating Evening Element present in the cis-element of DRE-Binding Factors (DREB) that target DRE and in turn drive the circadian rhythmic expression of *RD29*s. The circadian clock is an endogenous timekeeper, crucial to plant health as it coordinates growth and development with predictable diurnal environmental fluctuations and, correspondingly, to limit response to stimuli at physiological inappropriate times of day, thereby conferring improve growth and survival. Hence, a genetic pathway of plant circadian clock likely represents a mechanism i) contributes to tolerance to a broad range of environmental stresses, and ii) naturally adapt in to enhance stress tolerance mechanisms when exposed to deleterious condition [67]. Approximately 80% of transcripts in tomato and Arabidopsis plants have been identified as being under circadian control,[55,63-67] >50% of DRGs were found to be circadian clock genes. [64]. The majority of DRGs peak in the afternoon, a few hours ahead of cooler temperatures at night [58]. The caveat is that stress treatments may influence the diurnal rhythm of transcriptions. For instance, episodic severe droughts cause slight shifting of the clock period (<~1 hr), while reducing the level expression of several circadian clock oscillators such as *TOC1,LUX* and *PRRs* [67-

69].Though, continuous moderate drought does not reset the oscillation of core clock com-ponents [70] proposing that circadian regulation could ensure plants to grow during part of the diurnal cycle, while expressing stress tolerance mechanisms at times of the day when they are required most critically. Circadian oscillation emerges as an integral component of IST, contributing to a broad range of environmental stress tolerances and enhancing stress tolerance mechanisms when needed most critically. Overexpression of genes in circadian rhythm, as demonstrated in studies like that of the wheat expansin gene *TaEXPB23*, has shown normal growth and enhanced drought tolerance in Arabidopsis and Tobacco. On the other hand, plants over-expressing same under the 35S promoter showed abnormal growth [68]. Similar results were observed in studies done by [69] where overexpressing *Glyoxalase I (Gly I)* by circadian promoter was favored compared to constitutive promoter. This suggests that circadian rhythmic accumulation is a key factor in achieving drought-tolerant plants without compromising growth. Understanding the roles of circadian clock genes and associated DRGs is pivotal for improving crop performance under environmental stress conditions.

Future Perspective

In their natural environment, plants are constantly exposed to several biotic and abiotic stresses, which, particularly in agriculture, has resulted in significant output loss each year. For this reason, we are searching for a novel strategy to support genetic engineering or molecular breeding research to boost plants' natural defense capacities, particularly against drought stress, to improve production and survival. Until now, the studies of the mechanisms of drought tolerance by plants have found various explanations. For instance, the accumulation of secondary metabolites and defense compounds in plants was associated with enhanced resistance to biotic and/or abiotic stress [70]. This indicates that plants have the capacity for what can be described as

“memory”, also called priming [71]. According to [71], 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 [71]. 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 possible costs of priming are relatively fewer than direct defense [72]. 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. Understanding these mechanism and discoveries will then assist the delineation of the molecular mechanism of the plant defense responses and aid in generating genetically modified crops which will together help in improving the economic and environmental sustainability of agriculture.

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Table

Table 1: List of genes isolated from Arabidopsis and tested in major crops to confer Drought Tolerance.

Genes	Tested Plants	Stresses	Side Effects	Mode of actions	Ref.
AtHDG11	*Triticum aestivum* *Gossypium hirsutum*	Drought Salt	Reduced stomatal density	Transcription factor	[35, 73]
AtYUCCA6	*Solanum tuberosum*	Drought	Leaves area reduction	Auxin biosynthesis	[74]
AtWRKY30	*Triticum aestivum*	Heat, Drought	Unknown	Transcription factors	[75]
AtLOS5	*Zea mays* *Gossypium hirsutum*	Salt, Drought	Unknown	ABA biosynthesis	[76, 77]
Atδ-OAT	*Oryza sativa*	Drought, Salt	Unknown	Proline biosynthesis	[78]
AtNCED3	*Glycine max* L.	Drought	Photosynthesis Transpiration Stomatal conductance decrease	ABA biosynthesis	[79]
AtEDT1	*Medicago sativa* L.	Drought	Reduced stomatal density	Transcription factor	[80]
AtCBF1	*Solanum lycopersicum*	Drought	Fast stomata closure; dwarf phenotype	Transcriptional activator	[81]

Chapter 2

Engineering Induced Systemic Drought Tolerance in Arabidopsis

Introduction

Drought is one of major abiotic stresses limiting agriculture productions. To combat this, several biotechnology approaches have been employed to overexpress drought responsive genes and enhance drought tolerance in various crops [1-4]. However, an ectopic expression of DRGs shows a penalty on plant growth or development. Hence, recent studies have been exploring Induced Systemic Tolerance (IST) developed by Plant Growth-Promoting Rhizobacteria (PGPR), in order to develop an innovative, feasible strategy for upgrading plant defense capacity without yield losses. IST is a state of heightened defenses activated throughout the whole plant, providing enhanced tolerance toward various abiotic constraints, including drought, salinity, and extreme temperatures [5-9]. However, the molecular mechanism of IST activations and plant-PGPR interactions are still elusive.

While deciphering the mode of IST, we uncovered that IST-priming PGPR, *Paenibacillus polymyxa* CR1 can specifically induced the upregulation of two DRGs, namely *Response to Desiccation* (*RD*)*29A* and *RD29B* [10]. *RD29*s were identified initially as drought and stress-responsive genes. The promoter of *RD29A* contains ABA-responsive element binding factor (AREB/ABF) and drought-responsive cis-element binding protein/C repeat-binding factor (DREB/CBF) motifs, while that of *RD29B* harbors only AREB/ABF. Nonetheless, their function and activity have been uncharacterized [10-19]. Recently, our genetic studies have clearly demonstrated that *RD29*s are integral to

both drought tolerance and IST [9,20] through distinct pathways; *RD29A* is mainly cold- and drought-inducible, while *RD29B* is primarily induced by salt stress [18]. During IST activations, they differentially modulate drought-responsive TFs and target DRGs. Moreover, *RD29A* suppresses salinity responses and behaves as a negative regulator [20]. In line with this scenario, their knockout (KO) mutants showed higher vulnerability to drought compared to wildtype (WT) plants, with no alterations in stomata behavior or growth rates [9]. This positions *RD29A* and *RD29B* as feasible DRGs for engineering commercially viable drought-tolerant crops, enhancing survival in both optimal and stressed conditions. However, the constitutive overexpression of *RD29*s led to undesirable phenotypes in transgenic plants, perhaps because they are circadian clock genes being oscillatory expressed diurnally [20]. As an example, the constitutive overexpression of circadian clock gene *C-REPEAT BINDING FACTOR* (*CBF3*) under cauliflower mosaic virus (CaMV) 35S promoter exhibited enhanced stress tolerance in drought conditions, but penalized plant growth [21]. In comparison, when *CBF3* was introduced in *Arabidopsis* under the control of a promoter of *RD29A*, transgenic plants minimized growth inhibition, while retaining stress tolerance [22]. These observations have raised interest in identifying strong circadian promoters to customize the spatial and temporal overexpression of DRGs.

Therefore, this study was designed to address the utility of a strong circadian clock gene promoter (pCOR15A) to overexpress *RD29*s for drought resistance strategies. This approach will be a potential method to avoid unwanted side effects, especially growth and defense trade-offs, in the genetic engineering or molecular breeding approach for further upgrading stress tolerance/resistance in economically important crops and advancing our knowledge of plant IST for the innovative generating 'commercial-grade' drought tolerant plants, defined as enhancing growth and survival capacity in both resting and stressed conditions. To this end, a plant transformation vector was

constructed, and a series of experiments were conducted to make the transgenic plants from each construct.

Materials and methods

1. *Plant materials and growth conditions*

Arabidopsis thaliana plants (ecotype Columbia-0) were used to generate the transgenic lines. Seeds were incubated for 2 days at 4°C in the dark to break seed dormancy, then transferred to 22°C with a 12 h day cycle (100–120 μE/m^2/s) with 60–80 % relative humidity, and plants were grown for the various periods indicated.

2. *RNA extraction and analysis of quantitative real time-PCR (qRT-PCR)*

Total RNA was extracted from 4-week-old leaf by using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA qualities were assessed by agarose gel electrophoresis and NanoDrop (A260/A280 > 1.8 and A260/A230 > 2.0 [23]. RT reactions were performed using an oligo(dT) reverse primer and a qScript reverse transcriptase (Quantabio, Beverly, MA, USA). Quantitative PCR was performed with the PerfeCT® SYBR® Green Fast Mix® Reaction Mixes (QuantaBio, MA, USA) in the CFX96 TouchTM (Bio-Rad, Hercules, CA, USA) PCR system cycled 32 times using gene-specific primer sets (**Table 2.1**). The annealing temperature for the primer pairs was 55 °C. To determine the relative abundance of target transcripts, the sample cDNA was assessed with housekeeping genes, *POLYUBIQUITIN* (*UBC*), and the average threshold cycle (i.e., Ct) was normalized to that of UBC as $2^{-\Delta Ct}$ where $-\Delta Ct = (C_{t,gene} - C_{t,UBC})$.

3. *Agrobacterium-mediated transient expression*

Transient transformation mediated by Agrobacterium was performed according to the method described [24]. The individual constructs in *Agrobacterium tumefaciens* strain cells - *LBA4404* (GOLDBIO) were grown for 2 days. They were Agro-infiltrated in 6-weeks-old *Nicotiana tabacum* and *Solanum lycopersicum* (Micro-Tom var.) leaves for transient expression assay. For this purpose, the construct was grown in 5 ml LB media at 28 °C for 2 days with gentle agitation. Kanamycin was used at 50 μg/mL to select for the pCAMBIA1300-derived plasmids, while rifampicin at 5 μg/mL was used to select for agrobacteria. Then, the next day, culture 5ml of the overnight culture was grown in 25 LB media (with the same antibiotics, plus 20 μM acetosyringone). Bacteria was then precipitated (5,000 x g, 15 min) and pellet were resuspended in resuspension buffer (10 mM MgCl2, 10 mM MES [pH 5.6], 150 μM acetosyringone) to a OD_{600} 0.8. Then the mixture was incubated at room temperature for 2 h and infiltrated into underside of intact eaves using a 1 ml syringe without a needle

4. *Arabidopsis transformations*

Arabidopsis plants were grown till peak flowering, and their flower is dipped into an Agrobacterium suspension consisting of 5% sucrose and 500 μl per liter of surfactant Silwet L-77 [25]. Once the plants were floral dipped, they were grown for the seed settings. T_1 transgenic lines were selected by following the sandy way protocol with some modifications [26]. Approximately 20 mL of dry quartz sand was initially placed in the Petri plates. The sand was then saturated with around 10 mL of 1/4 MS Basal Salt media and contained hygromycin and kanamycin as an antibiotic. Then, the T_0 seeds were spread on the plates and were grown at 4°C for two days for vernalization. They were later switched to growth chamber wrapped in aluminum foil in a dark location overnight. Aluminum foils were removed after two days and were grown in the growth chamber in normal conditions. T_1 homozygous transgenic Arabidopsis plants genotypes were

verified by quantifying the expression of respective *RD29A* or *RD29B* genes by using qRT-PCR.

Results and discussion

1. *Identification of strong, circadian clock promoter*

To identify a strong promoter that controls an oscillatory pattern expression of circadian clock gene especially peaking at 3 pm in the afternoon as do *RD29*s, we surveyed the time resolved expression of known DRGs and circadian clock genes. To the end, we identified that *Cold-Regulated* (*COR*)*15A* exhibited the same diurnal expression pattern of *RD29*s but 10 times higher transcript levels (**Fig. 2.1**).

2. *Construction of plant overexpression vectors*

To generate plant overexpression constructs, the promoter region of *RD29A*, *RD29B*, *COR15A* and CaMV 35S were PCR amplified using specific primers (**Table 2.2**) and inserted into the 5′-region of multiple cloning sites of pCambia 1300 binary vector. In subsequent, the full-length genomic DNA sequence of *RD29A* (2.9 kb) and *RD29B* (2.5 kb) were also PCR amplified and were inserted into the 3′-region of multiple cloning sites of pCambia 1300 plasmid. These constructs were then transformed into *E. coli*; the double digestion of each construct with *BamH*I and *Kpn*I confirmed correct insertions (**Figs. 2.2A and B**). Insertions were further verified by the full sequencing and yielded a series of constructs including pRD29A:g*RD29A*, pRD29B:g*RD29A*, pCOR15A:g*RD29A*, p35S:g*RD29B*, pRD29A:g*RD29B*, pRD29B:g*RD29*B, pCOR15A:g*RD29B*, and p35S:g*RD29B* (**Fig. 2.3**). These constructs were again electroporated into *Agrobacterium tumefaciens LBA*4404.

3. *Screening of the T1 transgenic lines of the Arabidopsis plants*

After cloning different sets of vectors in *Agrobacteria*, the floral dippings were carried out to generate transgenic plants. *Arabidopsis* plants were grown till peak flowering, and their flower is dipped into an *Agrobacterium* suspension consisting of 5% sucrose and 0.5 mL/L of silwet L-77 [25]. Following the transformation, they were grown for seed (T_1 transgenic line) setting and selected each five genotypes through the sandy way protocol [26]. To further confirm the transformation of selected seedlings, the expression level of *RD29*s in each T_1 transgenic plant was determined by real time-qRT PCR analysis; at least 3 lines showing the higher overexpression levels were finally choose for the future analysis (**Fig. 2.4**).

4. *Construction of FLAG tag overexpression vectors and Arabidopsis transformation*

Since genes encodes proteins, we saw a need to understand the turnover time of RD29 proteins along with their circadian rhythm gene expressions. Thus, we also generated the full-length gene sequence of *RD29A* (2.9 kb) and *RD29B* (2.5 kb) fused with the flag tag sequence (5′-GACTACAAAGACGATGACGACAAG) at the 5′ end (**Fig. 2.5**). These constructs will allow us to determine the temporal level of RD29 translations and delineate if their circadian rhythmic expressions determine protein levels – constitutive and diurnal oscillatory.

5. *Circadian promoters confer drought tolerance in Solanum lycopersicum and Nicotiana benthamiana in Agrobacteria mediated transient gene expression analysis*

Agrobacterium-mediated transient expression assay is one of the important tools to analyze gene function for plants easily in a short period of time. Thus, to test our designed construct efficacy in conferring drought tolerance, we transiently expressed these genes by agro-infiltrating the *Solanum lycopersicum* - Micro-tom var. and *Nicotiana benthamiana* leaves. Once they were agroinfiltrated, they were exposed to drought by withholding water. Research findings indicate that genes introduced through agroinfiltration remain

detectable for up to twenty-seven days, with a peak observed on the 2nd day [34]. Thus, to ensure that gene expression level remains elevated while allowing plentiful time for the onset of observable drought stress symptoms, we opted to analyze the result after 7 days of agroinfiltration/drought stress. During the assessment, we observed control plants (infiltrated empty vector) were wilted and lodged, and leaves showed a yellowish phenotype compared to the transient expressed *RD29* genes construct plants in tomato and Nicotiana plants (**Figs. 2.6A and B**). Even though transiently expressed *RD29* genes driven by the 35S promoter were slightly better in drought tolerance compared to the vector control, it didn't perform better than another construct. Through this experiment, we could observe that RD29B promoters harboring different *RD29* genes were more tolerant to any other construct in tomato plants, whereas in tobacco plants, construct pRD29A:*gRD29A*, pRD29B:*gRD29A*, pCOR15A:*gRD29A* were more tolerant to drought stress. Even though, we used the same construct, we saw a difference in the drought tolerance variation in *Solanum lycopersicum* and *N. benthamiana.* It might be because the choice of plant species also affects the outcome of the transient gene expression experiment [28]. Nevertheless, the overall experiment indicates that genes driven by the native or strong circadian showed better plant drought stress tolerance. This further generates and holds the promise of the utility of circadian promoters. Although transient assays can provide some insights into the promoter's ability to regulate gene expression, these expression patterns might sometimes differ with stable events [29]. Thus, observing the stable transformation result for further analysis is still essential.

6. *Overexpression of* RD29A and RD29B *by using circadian promoter showed no visible side effect on vegetative growth of Arabidopsis thaliana under optimal growth conditions*

It was previously reported that *RD29A* and *RD29B* gene constitutively overexpressed by using 35s promoter showed stunted growth condition [19]. However, it is not clear

whether *RD29A* and *RD29B* gene overexpression using circadian promoter affects plant growth and development or not. To address this, phenotypes were compared among *RD29A* and *RD29B* overexpression lines and Col-0 (WT) during their vegetative growth period (**Fig. 2.7**). Under optimal growth conditions, as expected p35s: RD29A construct showed stunted growth. Conversely, p35s: gRD*29B* did not display significant stunting, however still showed relatively smaller size compared to the wild type. Notably, lines overexpressing *RD29A* and *RD29B* under the control of native or strong circadian promoters showed no discernible phenotypic difference compared to the wild type. This phenomenon might be attributed to the modulation of the gene over expression patterns in accordance with circadian rhythm pattern, which we hypothesized key to the generating drought tolerant plants without having growth side effects.

Conclusion

Recently identified as PGPR responsive genes - *RD29A* and *RD29B* have long been considered as DRGs and validated that play intrinsic roles in IST activations and defense responses against drought. However, the constitutive overexpression of these genes could still engender side effects and result in stunted growth. It might be because they are circadian clock genes, and dysregulation of their circadian pattern expression might hinder plant growth. To find a novel promoter, we identified a strong circadian COR15A promoter, which is expressed at a higher level compared to the native circadian RD29A and RD29B promoter. Moreover, the expression pattern of the COR15A was similar to these *RD* genes, but transcripts were in the higher fold. This gene expression pattern raised the hypothesis that using such a strong circadian promoter might replicate the IST molecular mechanism induced by PGPR *P. Polymyxa* CR1, wherein such genetically engineered plants will be able to confer drought without growth tradeoff. Thus, we chose four different promoters: native circadian pRD29A and pRD29B, strong circadian

promoter pCOR15A, and strong constitutive promoter p35S harboring either *RD29A* or *RD29B* gene. Altogether, we generated 8 different sets of plant transformation vectors, i.e., pRD29A:g*RD29A*, pRD29B:g*RD29A*, pCOR15A:g*RD29A*, p35S:g*RD29A*, pRD29A:g*RD29B*, pRD29B:g*RD29B*, pCOR15A:g*RD29B*, p35S:g*RD29B* to genetically engineered plants to test our hypothesis. Along with that, we performed transient gene expression analysis in different plants, which further hints at the circadian promoter's ability to enhance drought tolerance better than the constitutive promoter and its wild-type plants. Through the stable transformation in Arabidopsis plants, it showed that overexpression of *RD29A* and *RD29B* by using circadian promoter showed no visible side effect on growth compared to WT of Arabidopsis plants under optimal growth conditions. However, their drought tolerance performance is still yet to be explored. Indeed, till now these findings has suggest the utility of circadian promoters to replicates the IST molecular mechanism of the PGPR *P. Polymyxa CR1*, thus opening avenues for generating economically viable drought tolerant crops. These holds promise for advancements aimed to curb the global drought challenges in agriculture.

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Figures

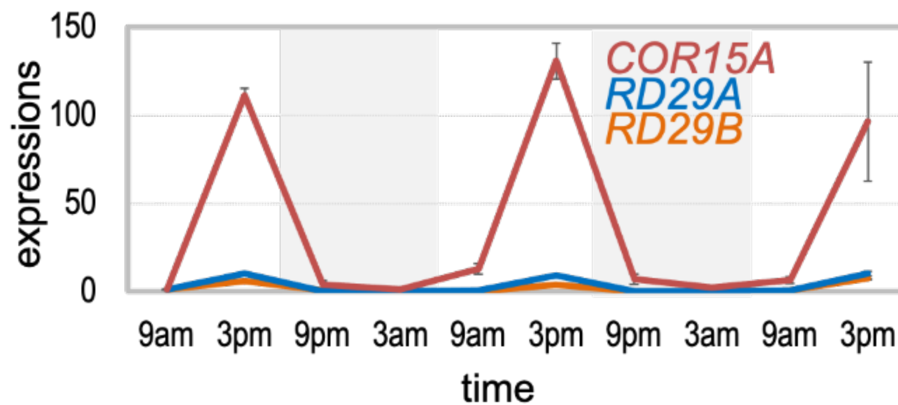

Fig 2. 1 : Identification of a strong circadian promoter.

Time-resolved qRT-PCR analysis of *RD29A*, *RD29B* and *COR15A*. Total RNAs were prepared from Arabidopsis plant leaves at 9am, 3pm, 9 pm and 3 am. Values were normalized to the expression of *UBC* gene (means ± SD, $n = 3$).

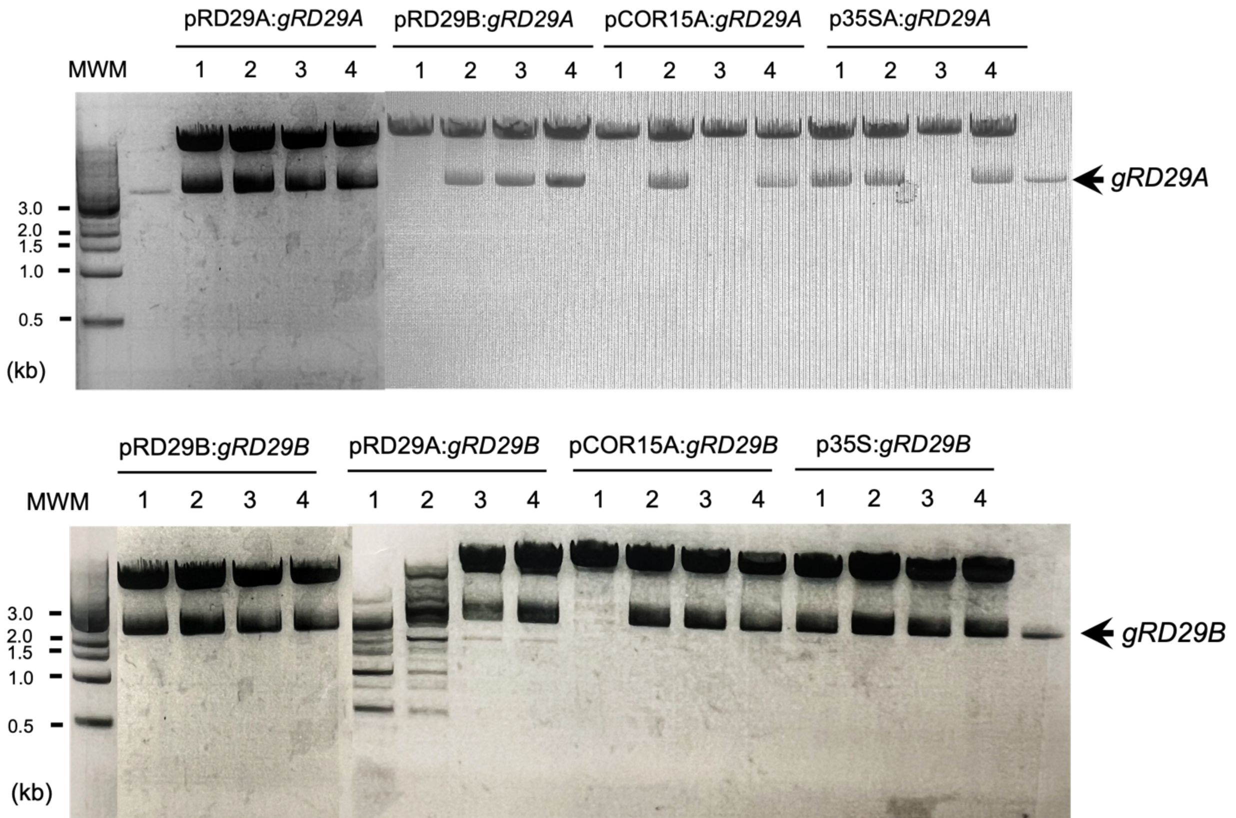

Fig 2. 2 : Preparation of plant transformation constructs.

Restriction enzyme digestion of pCambia harboring *gRD29A* (Upper) and *gRD29B* (Down) with *BamH*I and *Pst*I with *BamH*I and PstI. The arrow indicates the *gRD29s* loaded as control. 1, 2, 3, 4 represent the different colonies. MWM represent the 1Kb DNA ladder.

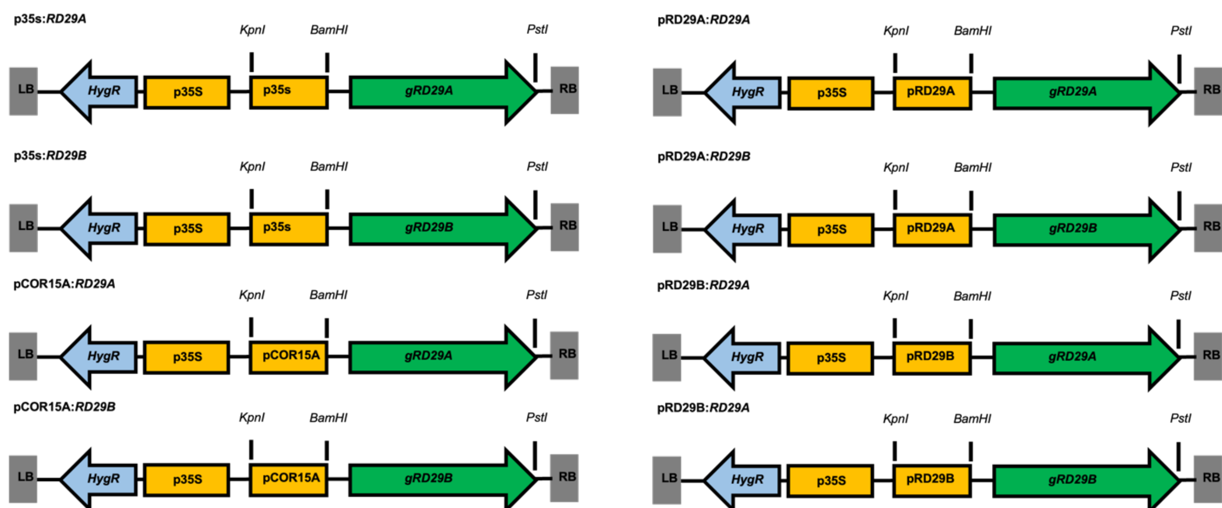

Fig 2. 3: Schematic representation of plant transformation vectors.

The T-DNA with left border (LB) and right border (RB) is represented by grey box. The different promoters such as pRD29A, pRD29B, p35S and pCOR15A are indicated by orange arrows. The genomic region of *RD29A* and *RD29B* are represented by green box. The hygromycin resistance gene (HygR) is represented by blue box. The restriction sites used for the cloning purpose *Kpn*I, *BamH*I and *Pst*I on vector cassettes are indicated by line.

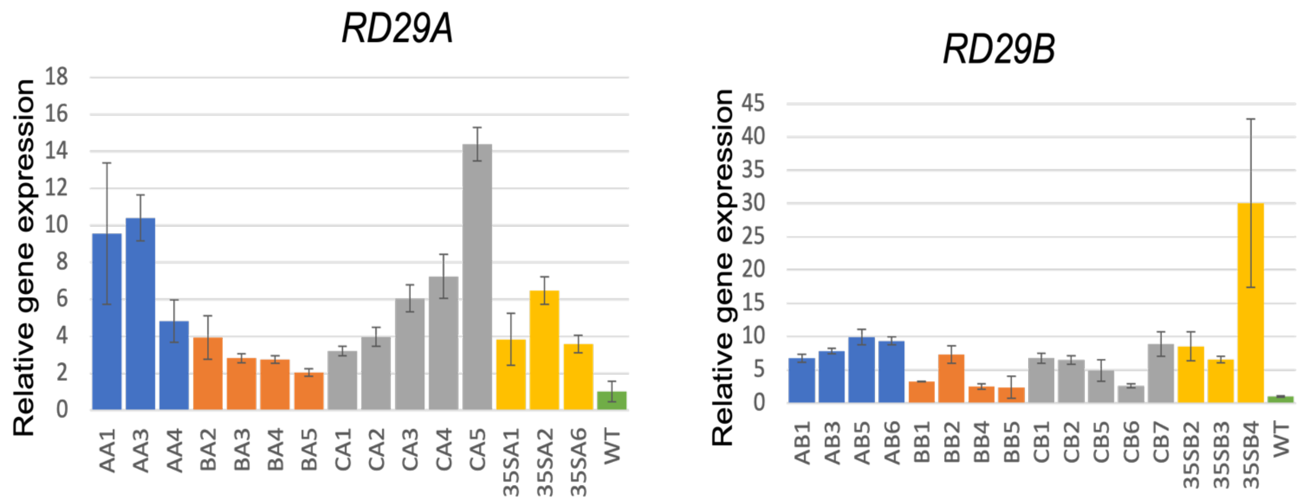

Fig 2. 4 : Relative gene expressions of *RD29*s in transgenic lines.

qRT-PCR analyses of level *RD29A* (***A***) and RD29B (***B***) mRNAs under the control of native promoter, pRD29A (pA) and pRD29B (pB), strong constitutive promoter, CAMV-p35S (p35S), or strong circadian promoter- pCOR15A (pCOR).

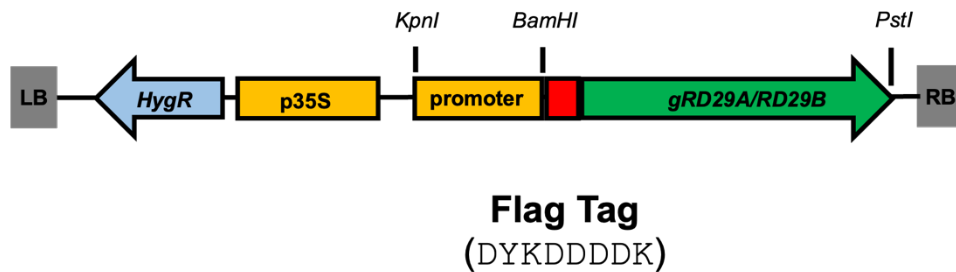

Fig 2. 5 : Schematic representation of plant transformation vectors in binary vector pCAMBIA 1300 with Flag tag sequence.

The T-DNA with left border (LB) and right border (RB) is represented by grey box. The different promoters box is represented by orange box. The *RD29A* and *RD29B* gene are represented by green box. The hygromycin resistance gene (HygR) is represented by blue box. Flag tag sequence is represented by red box. The restriction sites used for the cloning purpose *Kpn*I, *BamH*I and *Pst*I positions on vector cassettes are indicated by line.

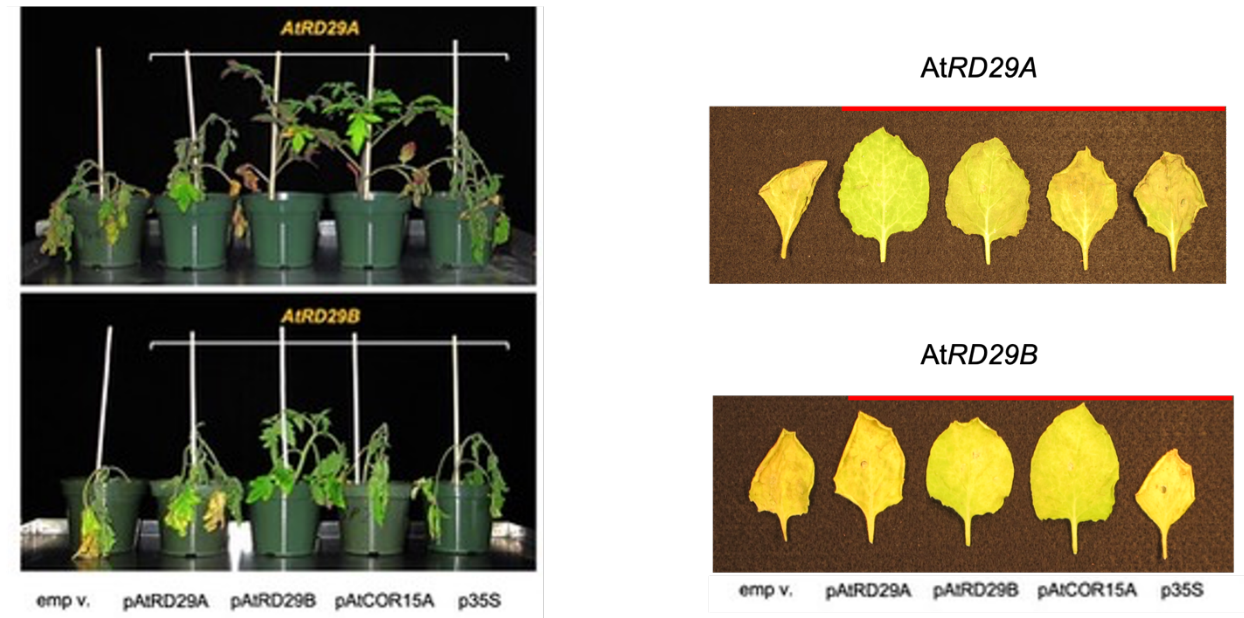

Fig 2. 6 : Drought Tolerance of agroinfiltrated *Solanum lycopersicum* and *Nicotiana benthamiana* leaves.

The underside of *Solanum lycopersicum* (Left) and *N. benthamiana* (Right) leaves were agroinfiltrated and were exposed to drought for 7 days. Empty vector (emp v.).

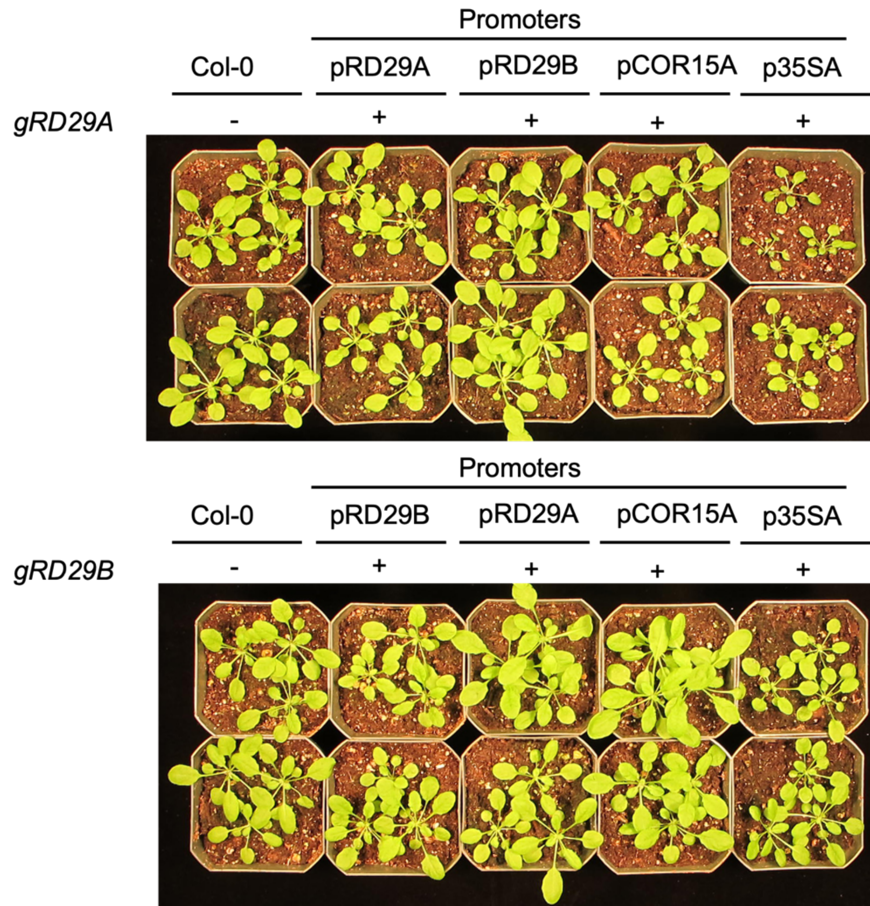

Fig 2. 7: Phenotypic analysis of transgenic Arabidopsis line overexpressing *RD29*s under optimal conditions.

Tables

Table 2. 1: Oligonucleotides used for cloning

Genes	Directions	Sequence (5′-3′)
gRD29A_BamHI	Forward	ATA TAT GGA TCC ATG GAT CAA ACA GAG GAA CCA
gRD29A_NdeI	Reverse	ATA TAT CTG CAG AAT ATG CTC TCA TCC TAG AAA T
gRD29B_BamHI	Forward	ATA TAT GGA TCC ATG GAG TCA CAG TTG ACA CG
gRD29B_NdeI	Reverse	ATA TAT CTG CAG TCT TAT CCA AAA AAG CAA ATA TTT
p35S_KpnI	Forward	ATA TTA GGT ACC ACA TCA ATC CAC TTG CTT TG
p35S_BamHI	Reverse	ATA TTA GGA TCC CCA TGG AGT CAA AGA TTC
pCOR15A_KpnI	Forward	ATA TTA CTA ACA TGT AAG TTT TTG TTA
pCOR15A_BamHI	Reverse	GGT ACC AGA TGT GAG AAT AAA AAG AAGT
pRD29A_Kpn1	Forward	GCT ATC GGT ACC AGA TTT GGG GTT TTG CTT TTG
pRD29A_BamHI	Reverse	GCT ATC GGA TCC TCC AAA GAT TTT TTT CTT TCC
pRD29B_KpnI	Forward	GCT ATC GGT ACC CGT AAT TTT CTA GAT CCG TCT TGG
pRD29B_BamHI	Reverse	GCT ATC GGA TCC TCC AAA GCT GTG TTT TCT C

Table 2. 2 : Oligonucleotide used for RT-PCR

Genes	Directions	Sequence (5′-3′)
UBC At5g25760	Forward	CTG CGA CTC AGG GAA TCT TCT AA
	Reverse	TTG TGC CAT TGA ATT GAA CCC
RD29A At5g52310	Forward	ATC GAT GCA CCA GGC GTA A
	Reverse	TGC ATC GTG TCC GTA AGA GG
RD29B At5g52310	Forward	ACG AGC AAG ACC CAG AAG TT
	Reverse	AGG AAC AAT CTC CTC CGA TG

Chapter 3

Preparation and characterization of the deglutathionylation activity of Arabidopsis thaliana glutaredoxin 480

Introduction

Glutaredoxins (GRXs) are heat-stable oxidoreductase that use reduced glutathione (GSH) to metabolize thiol-disulfide exchanges with protein-disulfides, namely 'deglutathionylation' [1-3]. GSH is the most prevalent nonprotein thiol in plants, playing a crucial role in maintaining cellular redox homeostasis under different ecological conditions. Under oxidative stress, it reduces reactive oxygen species (ROS) and other peroxides by providing its electron (e−, H+), and is subsequently oxidized to a disulfide form (GSSG). GSSG then binds and forms a mixed disulfide with a protein cysteine (Cys) which is S-glutathionylation (PSSG) that protects proteins from irreversible oxidation damages. When the stress is over, GRXs deglutathionylate and restore the activity of PSSG [2] This posttranslational modification (PTM) in turn regulates redox signaling and various cellular processes in photosynthesis, iron homeostasis, sulfur and nitrogen assimilations, stress adaptation, and innate immune response in plants [4-6].

GRXs are classified into three classes: I (CXXC/S), II (CGFS) and III (CCXC/S). Classes I and II are ubiquitous in both prokaryotes and eukaryotes, while class III is exclusive to the land plant species [7]. For instance, a model plant system Arabidopsis consists of 32 GRXs: 5 class I, 6 class II and 21 class III (**Table 1**). All class I appears to be active enzymes, capable of using GSH to reduce dehydroascorbates, but only GRXC1 and -C5 can use the iron-sulfur (Fe-S) cluster as a cofactor. Instead, all class II moves electrons (e−, H+) from the Fe-S cluster to target proteins. Together, class I and II GRXs involve in seed, embryo and flower developments, photosynthesis, and respiration, as well as stress

adaptations and defense responses. On the other hand, only few class III has been biochemically characterized, perhaps due to their carboxyl terminal hydrophobic tail (G/K)A(L/I)W(L/I/V/F) that hinders the expression and solubilization of recombinant proteins [8]. Interestingly, the same C-terminus tail may allow GRXs to bind and activate TGA transcription factors (TFs), belonging to a basic leucine zipper family that functions in various signaling pathways involved in hormone, reactive electrophilic species, and redox cues [9-11].

Most notably, ROXY19 (also known as GRX480) binds and controls TGA2 and TGA6, which convey both nonexpressor of pathogenesis-related protein 1 (NPR1)-dependent salicylic acid (SA) defense gene expressions [12], and cyclophilin 20-3 (CYP20-3)-dependent 12-oxophytodienoic acid (OPDA) responsive gene expressions [11, 13]. SA and OPDA responsive genes in turn activate and calibrate defenses against various environmental stresses, as well as growth and development in plants [14,15]. Level *GRX480* mRNA is upregulated in response to SA that is induced essentially by biotrophic pathogens, as well as OPDA when produced in response to necrotrophic microbes, pests and abiotic stresses including salinity, excess oxidants and light, and mechanical wounding [16]. In line with this scenario, SA and OPDA signaling systemically induce GSH accumulations, and build up cellular reduction potential [13, 17]. The enhanced redox capacity (increased GSH/GSSG ratios) in turn triggers retrograde directional signaling to turn on nucleus gene expressions. Therefore, it is plausible to speculate that GSH donates electron (e–, H+) to GRX480 and stimulates the GRX480-TGA2/6 TF system, coordinating the expression of a subset of SA- and OPDA-responsive gene expressions, in particular those involved in general defense responses such as *HEAT SHOCK PROTEIN 70.6* (*HSP70.6*) and *HSP17* [11, 13, 18, 19].

Collectively, future investigations of GRX480 will help delineating regulatory gaps in plant hormone signaling, and their crosstalk that activates unique and conserved (general) defense mechanisms to coordinate ultimate recovery systems. However, the biochemical activity of GRX480 is still unknown. To better understand its roles, especially, its binding dynamics with TGA TFs in conjunction with SA/OPDA-dependent GSH accumulations, we here have successfully enriched and purified recombinant GRX480 in help of a highly soluble maltose binding protein (MBP) as a solubility enhancer and validated its enzymatic activity. Measurement of the deglutathionylation kinetics of GRX480 toward S-glutathionylated bovine serum albumin (BSA) determined its apparent Km values as 6.4 μM, a considerately lower value than other class I GRXs (**Table 1**), indicating that GRX480 is a highly active, functional enzyme.

Materials and methods

1. PCR and molecular cloning

The coding sequence of GRX480 (at1g28480) was PCR cloned into four *E. coli* expression vectors; pET28a and pET28c with N- and C-terminus 6xHIS (Novagen) respectively, pGEX-4T-1 harboring N-terminus GSH-S-transferase (GST, Cytiva), and pKLD66 containing N-terminus 6xHIS tagged maltose-binding protein (MBP) [45]. Total Arabidopsis RNA was prepared using TRIzol reagent (Invitrogen) and the Direct-zol RNA Kit (Zymo Research) according to the manufacturer's instructions. The quality of RNA was assessed by agarose gel electrophoresis and NanoDrop (A260/A280 > 1.8 and A260/A230 >2.0; [20]. cDNA was generated by an oligo(dT) reverse primer and qScript reverse transcriptase (Quantabio). PCR was performed with Q5 High-Fidelity DNA Polymerase (New England Biolab) and the CFX96 Touch™ PCR system (Bio-Rad), using

specific primer sets (**Table 3.2**). The annealing temperature for all primer pairs were 55 °C. Each construct was then heat-shock transformed to *E. coli* BL21 (DE3). Positive clones were selected by appropriate antibiotics, and inserts were confirmed via the double digestion with a pair of restriction enzymes, and plasmid sequencing (Eurofins Genomics).

2. *Expression of recombinant GRX480 in E. coli*

A single colony harboring *GRX480* in different expression vectors was grown in 5 mL Luria Broth (LB) media overnight at 37 °C under appropriate antibiotics as a seed culture. The seed culture was diluted into 50 mL LB media for initial expression trails or into ~ 1 to 5 L LB media to carry out further protein purification processes. Each bacteria culture was grown to reach optical densities (OD_{590}) at ~0.6 and induced by various concentrations (0.01-0.1 M) of isopropyl ß-D-1-thiolgalactopyranoside (IPTG) in a shake incubator (225 ppm) for 3 to 16 hr at 19 or 37 °C.

For the initial expression trail, 5 mL of *E. coli* cells collected with/without IPTG induction at various concentrations, times and temperatures were pelleted down by a centrifugation and resuspended in 50 μL of NuPAGE™ LDS sample buffer (Thermo Scientific) with β-mercaptoethanol as a sample. Samples were heated at 100 °C for 10 min, centrifuged down to collect each aliquot that were subsequently loaded (12 μL) on SDS-PAGE for the separation. Gels were visualized by Coomassie G-250 staining solution (VWR).

3. *Affinity purification of recombinant GRX480*

For the protein purification process, *E. coli* cultures were pelleted down by centrifugation at 4 °C and resuspended in loading buffers; 50 mM $NaPO_4$ buffer (pH 7.5) containing 150 mM NaCl for HIS-tagged proteins, and 50 mM TRIS-HCl buffer (pH 7.5) containing 150

mM NaCl for GST-fused proteins. *E. coli* cell walls were broken down by a sonicator (Ultrasonic Processor), and subsequently removed by centrifugation at 13,000 g. The total protein extracts were then subjected to loading buffer equilibrated Ni-NTA resin (Thermo Fisher Scientific) for HIS-tagged proteins and GSH resin (Thermo Fisher Scientific) for GST-fused proteins and incubated for one hr with continuous agitations. The resin slurry was then gently loaded into Econo-Pac® chromatography columns (Bio-Rad) and resin-unbound protein was collected as flowthrough. Resin nonspecifically bound proteins were washed through with 4-time volumes of resin by loading buffers with/without 50 mM imidazole. The resin bound proteins were then eluted by the loading buffer with increasing imidazole concentrations up to 500 mM, or 10 mM GSH.

4. *Cleavage of HIS-MBP tag by TEV protease*

Following the Ni-NTA resin purification of recombinant HIS-MBP tag fused GRX480, HIS-MBP tag was removed by a cleavage reaction using the Turbo TEV protease (Promega) at 30°C for 4 hr, according to the manufacturer's instruction. TEV protease was added based on the quantity of purified recombinant proteins. We used 2µl of TEV protease protein per 20 µg of the fusion protein. After cleavage, HIS-MBP tag were removed by the second Ni-NTA column purification.

5. *SDS-PAGE and Western Blots*

SDS-PAGE were prepared and ran using Mini-PROTEAN® Tetra Handcast Systems (Bio-Rad) at percentages of acrylamide ranging from 12 to 13%. For western blots, the samples separated on the SDS-PAGE were electroblotted onto the Polyvinylidene Difluoride membrane (Millipore). The resulting blots were probed with monoclonal HIS-antibody (1:10000, Invitrogen) for 2 h. The blots were then incubated with the secondary antibody conjugated with horseradish peroxidase (1:5000) for 2 to 4 hr and visualized by

an enhanced chemiluminescence kit (GE Healthcare) and a luminescent image analyzer (ImageWuant LAS-4000, GE Healthcare).

6. Enzymatic activities of recombinant GRX480

Enzymatic activity of GRX480 was measured using the glutaredoxin fluorometric assay kit (Cayman) following the manufacturer's instruction. For each assay, eosin standard curve was calculated, and GRX positive and blank negative controls were tested along with a sample, GRX480 protein. Briefly, GRX480 was incubated with electron (e^-, H^+) donor NADPH, glutathione reductase, and a GRX substrate, eosin-GS-BSAs, and its activity was measured every min for 45 min by realizing eosin-GSH using the CytationTM3 Multi-Mode Reader (BioTek) at excitation and emission wavelengths at 520 nM and 560 nM, respectively.

Results and discussion

1. *In silico modeling suggests that GRX480 is enzymatically active and binds to GSH.*

As a first step to understand if GRX480 is enzymatically active, we employed the I-TASSER (Iterative Threading Assembly Refinement) server [21] to predict the presence and/location of its active binding sites/residues to GSH [22]. The I- TASSER first identified the structural templates of GRX480 based on the protein database (PCB) by multiple treading approach, LOMETS (Local Meta-Threading Sever) with full-length atomic models constructed by iterative template-based fragment assembly simulations [23]. Function insights of the GRX480 were then derived by re-threading the 3D models through protein function database BioLip [24] and estimated to bind GSH with a high

confidence(c) score of 0.742 at cysteine (C)52, proline (P)101 and glycine (G)112 residues. In added, these active site residues are highly conversed across the enzymatically active GRXs (Fig 1), further supporting that GRX480 is likely able to bind GSH, and an active enzyme.

2. *E. coli expressions of GRX480 using the pET system are ineffective*

To substantiate the in-silico prediction, we PCR cloned a coding sequence of *GRX480* to the pET vector system harboring the N-terminus (pET28a) and C-terminus (pET21c) HIS tag. The constructs were then transformed into *E. coli* BL21 (DE3) cells, and induced with several IPTG concentrations (e.g., 100mM; **Fig 3.2**). However, the expected size (~16 and ~15 kDa, respectively) of recombinant (re)GRX480 was not detectable in SDS/PAGE (**Fig 3.2, lanes I and II**), as suggested previously [8]. The pET-GRX480 system could produce as low concentrations of reGRX480 as detectable only by the protein blotting (PB) assays using the monoclonal anti-HIS antibody (**Fig 3.3**). We thus scaled up cell culture volumes up to ~ 5 L to enrich reGRX480, but its yields remained low and impure after the Ni-NTA resin affinity chromatography (**Fig 3.4 A and B**). To the end, we concluded that the pET vector system is not effectual for reGRX480 expressions and purifications, regardless of HIS tag positions (N- and C-terminus), as well as IPTG concentrations (1 to 100 mM) and its incubation times (3 to 16 hr) and/or temperatures (19 or 37 ^{o}C).

3. *N-terminus fusion of HIS-tag GST did not improve the induction of GRX480.*

We hypothesized that the difficulty of GRX480 expressions in the pET vector system is due to its C-terminus domain which insolubilizes proteins in *E. coli* [8]. To improve the solubility of proteins, we subcloned *GRX480* into pGEX-4T-1 harboring N-terminus HIS-tagged GST to generate GST-labeled GRX480. GST is one of effective, soluble fusion tags in BL21 (ED3). These fusion proteins or tags acts as chaperones, assisting their protein folding which in turn helps in solubilization of the recombinant proteins and also helping

in ease of its purification [25]. However, similar to the pET system, the pGEX-4T-1 vector yielded negligent level GRX480 inductions (**Fig 3.2, lane IV**). Upon the purification process, little protein of expected size (~39 kDa) was detected in the elution fraction (**Fig 3.4 C**). GST is often a poor solubility tag when compared to other commonly fusion partners, rendering the target protein production into inclusion bodies [26].

4. *N-terminus fusion of MBP drives the enhanced induction of GRX480.*

Next, we examined the efficacy of another solubility enhancer, HIS-tagged maltose binding protein (MBP) using the pKLD66 system [27] . Several studies demonstrated that N-terminus MBP fusions improve the solubility and yield of recombinant proteins [28]. Indeed, IPTG application were able to induce the sufficient level, expected size (~56 kDa) of HIS-MBP-reGRX480 (**Fig 3.2, lane III**) which was subsequently purified by the Ni-NTA affinity column using the salt, imidazole, elution (**Fig 3.5A, lane 5**). Here, reGRX480(13kDa) and MBP proteins (42kDa) were fused by a poly-linker that contains a Tobacco Etch Virus (TEV) nuclear-inclusion-a endopeptidase recognition site (ENLYFQ^G) [27]. Hence, MBPs were cleaved at 30°C (**Fig 3.5B, lane 2**) and subsequently removed by again the Ni-NTA chromatography (**Fig 3.5B, lane 3**). The yield of reGRX480 prepared by the pKLD66 vector was significantly higher, likely sufficient for enzymatic assay, compared to other vector systems tested such as pET and pGEX-4T-1 vectors.

5. *Arabidopsis GRX480 is enzymatically functional, transferring electrons (H+, e−) from GSH to reduce S-glutathionylated BSA.*

GRXs are known to reduce PSSG using GSH as an electron donor (i.e., deglutathionylation), which can be measured using an artificial substrate such as eosin-labeled S-glutathionylated bovine serum albumin (GS-BSA). Using the GS-BSA substrate, we characterized the deglutathionylation ability of GRX480. In this assay, the 2-step reaction was carried out. GRX480 metabolized electron (H^+, e^-) transfers from GSH to,

and oxidized, the eosin-GS-BSAs substrate, producing eosin-GSH and BSA-SH. Eosin-GSH, a fluorescence product, then was detected with excitation at 520 nm and emission 560 nm once every minute for 45 min. In the second step, GRX480 is reduced, recharged by GSH to produce GSSG, which is recycled via a couple of reactions via GSH reductase and NADPH. These reactions continue, and corresponding signals (relative fluorescent units, RFU) increase in a linear relationship with the oxidoreductase activity of GRX480, measured up to 45 min and calculated its activity as 0.39 μM per min (**Fig 3.6A**). To further characterize the activity of GRX480, we examined it under the varied concentration of eosin-GS-BSA substrates (4 to12 μM), describing its apparent *K*m values as 6.4 μM, (**Fig 3.6B**). This value is considerately lower than other class I GRXs characterized (**Table 1**), revealing for the first time that GRX480 is a highly active, functional enzyme.

Conclusion

In this study, we successfully prepare reGRX480, a class III GRX, and validated −for the first time− its oxidoreductase activity (Figs 4 and 5). GRX480 is a TRs differentially up- and downregulated by jasmonate, SA and ABA hormones [11-13] and in turn targets and regulates TGA2, TGA5 and TGA6, key TGA TFs of OPDA and SA signaling in plant defense responses [9-11, 29-31]. In that context, previous studies from our and other groups showed that activation of OPDA and SA signaling also stimulates cellular sulfur assimilations leading to GSH (cofactor) accumulations, independent of oxidative stress signaling [13, 17, 31]. Hence, ***in our model,*** when the photosystem I (PSI) antenna captures solar energy in resting states, it prompts a chain reaction of electron (H^+, e^-) transfers that elicits TRX-based redox regulations in controlling energy (sugar) conversions and consumptions. By contrast, under stressed conditions, defense hormones OPDA and SA are accumulated and, in subsequent, stimulate electron (H^+, e^-) transfers from TRXs to

their key regulators such as CYP20-3 and NPR1. Reduction of NPR1 allows its re-entry into the nucleus and binding/activating TGA TFs, leading to the induction of *GRX480* [32] GRX480 then form a ternary complex with TGA/NPR1, and negatively regulates JA signaling [12]. On the other hand, the activation of CYP20-3 stimulates the formation of Cys synthase complex (CSC), leading to Cys biosynthesis and producing thiol metabolites (e.g., GSH) [17, 31, 33-35]. The enhanced reduction potential (i.e., increased GSH:GSSG ratios, [13,36] , then escalates the crosslinking of GSH with GRX480 [29]. GRX480-medaited PTM (deglutathionylation) then regulates the cellular activity and mechanism of target proteins [37] such as TGA TFs, so that temporally rearranges the expression of OPDA responsive genes, actuating defense responses against various ecological constraints such as microbial pathogens, salinity, excess oxidants and light, as well as mechanical wounding [13, 18]. In agreement with our working model, a number of studies have corroborated the positive correlation of sulfur assimilation (GSH productions) with plant resistance to a wide range of pathogenic microbes, insects and herbivores (reviewed in 11), a phenomenon termed sulfur-induced resistance (SIR) [38]. Although metabolic details underlying SIR remain to be identified, cellular contents of GSH may be among the crucial factors linking sulfur nutrition. However, the mode how GRX480 utilize GSH in its TGA interactions, as well as defense signaling and gene expressions are still elusive. The finer aspect of dynamic relationship between OPDA, SA and GSH, GRX480 and redox changes will have to be explored in future work.

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Figures

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AtGRX480   RRGCCMCHVVRRL-LLGL---GVNPAVLEIDEERED--EVLSELENIGVQGGGGTVKLPAVYVGGRLFGGLDRVMATHISGELVPILKEVGALWL ------------------137
hGRXC2     KTSCSYCTMAKKL-FHDM---NVNYKVVELDLL-EYGNQFQDALYKM-----TGERTVPRIFVNGTFIGGATDTHRLHKEGKLLPLVHQCYLKKS KRKEFQ------------164
ZfGRX2     KTTCPYCKMAKGV-FNEI---GATYKVVELDEH-NDGRRLQETLAEL-----TGARTVPRVFINGQCIGGGSDTKQLHQQGKLLPLIEQCRPCCL NMTPEGSGNSQNQPHQ-134
ScGRX2     KTYCPYCKATLSTLFQELNVPKSKALVLELDEM-SNGSEIQDALEEI-----SGQKTVPNVYINGKHIGGNSDLETLKKNGKLAEILKPVFQ--- ------------------129
ScGRX1     KTYCPYCHAALNTLFEKLKVPRSKVLVLQLNDM-KEGADIQAALYEI-----NGQRTVPNIYINGKHIGGNDDLQELRETGELEELLEPILAN-- ------------------118
PtGRXS12   KTWCSYSSEVKSL-FKRL---NVDPLVVELDELGAQGPQIQKVLERL-----TGQHTVPNVFIGGKHIGGCTDTVKLYRKGELEPLLSEANAKKS QG----------------112
AtGRXC5    KTWCSYCTEVKTL-FKRL---GVQPLVVELDQLGPQGPQLQKVLERL-----TGQHTVPNVFVCGKHIGGCTDTVKLNRKGDLELMLAEANGKNG QS----------------113
CreGRX2    KTYCPYCVKAKNA-LNQFI--AGKYTVVELENR-ADCDAMQDALLDI-----TGGRSVPRVFINGKFLGGGDDTAAAASNGTLEKLLQEAGAL-- ------------------107
wGRX       KSYCPFCVQVKKL-FTQL---GASFKAIELDTE-SDGTEIQSALAEW-----TGQRTVPNVFINGKHIGGCDDTIALNKGGKLVALLTEAGAISG SSSKTTVTPLEHHHHHH171
PtGRC1     KTYCGYCNRVKQL-LTQV---GASYKVVELDEL-SDGSQLQSALAHW-----TGRGTVPNVFIGGKQIGGCDTVVEKHQRNELLPLLQDAAATAK NPAQL-------------117
rbGRX      KTYAGYCSRVKKL-FDQL---GARYQTIELDQE-SDGDAIQAALLQW-----TGQRTVPNVFIGGKHIGGCDSVMEKHRDGKLVPMLTECGAIAI ESTA--------------129
           :  .  .  .    :  .        .::::       .   *         *   .:* ::: *   :**           . *   ::
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Fig 3. 1: Alignment of the Arabidopsis GRX480 protein with the enzymatically active glutaredoxin from various species.

Green boxes represent the conserved GSH binding sites in comparison with AtGRX480 predicted by ITASSER modeling, Yellow represent the active GSH binding sites similar to the predicted GSH binding sites of AtGRX480, Blue color represent the unique GSH binding sites present in AtGRX480 predicted by ITASSER modeling. AtGRX480/GRXC5: Arabidopsis Glutaredoxin 480/C5, hGRXC2; Human Glutaredoxin C2; ZfGRX2: Zebra fish glutaredoxin2, ScGRX1/2: Yeast Glutaredoxin1/2; CreGRX2: Algal Glutaredoxin2; wGRX: wheat Glutaredoxin; PtGRC1: Poplar Glutaredoxin C1; rbGRX: Buckwheat Glutaredoxin.

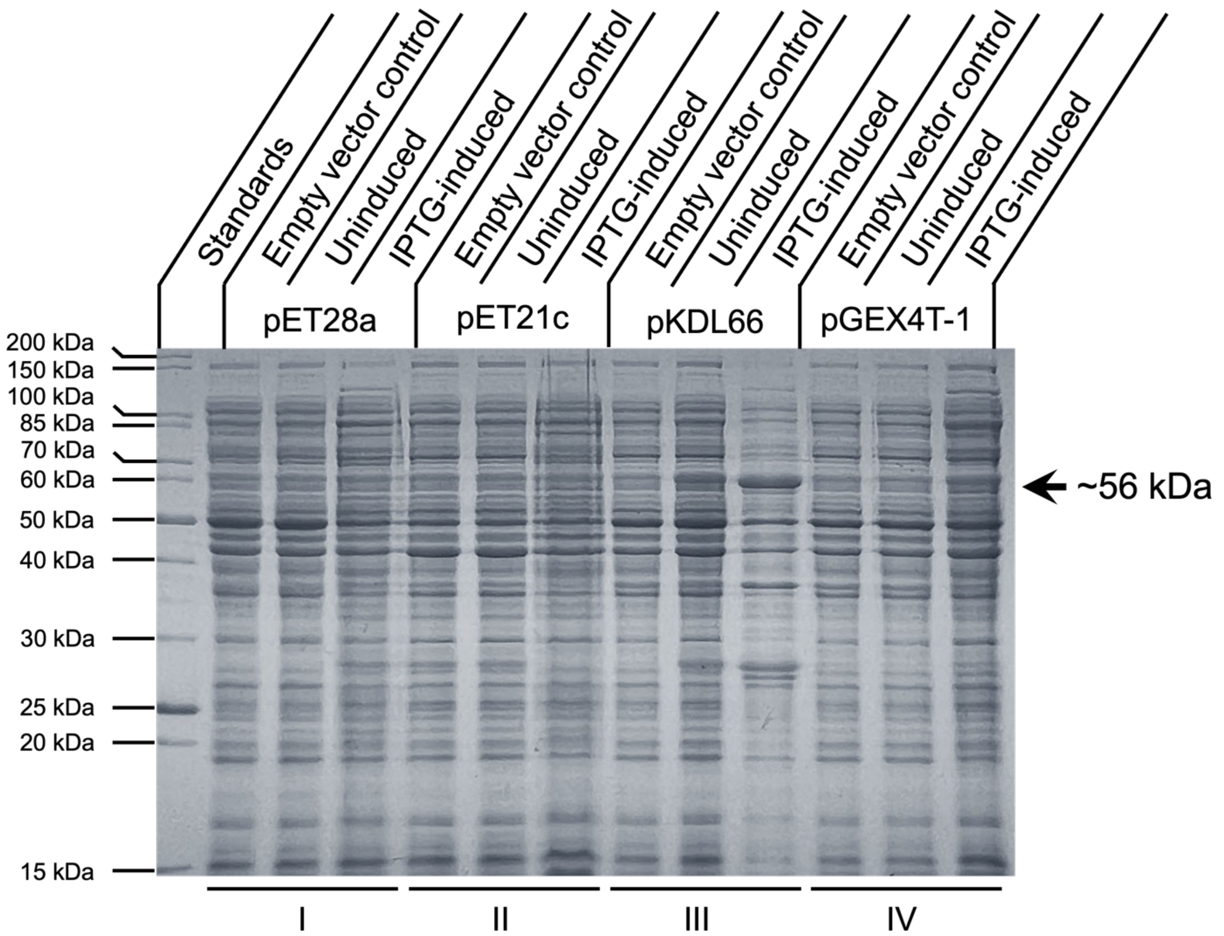

Fig 3. 2: Comparison of reGRX480 expression using different expression construct.

For the analysis reducing 13 % SDS-PAGE was employed. The gel lanes were loaded as follows; Standard: PageRuler™ Unstained Protein Ladder; Empty vector control: total protein from E. coli BL21- cells containing native expression vector; Uninduced: total protein of E. coli BL21 cells with expression vector-GRX480 before IPTG induction; IPTG-Induced: total protein of E. coli BL21 cells with expression vector-GRX480 after induction by IPTG(100mM) at 37◦C for 4h. Lane I represent expression vector(pET21a)-GRX480; Lane II represent expression vector (pET21c)-GRX480; Lane III represent expression vector (pGEX4T-1)-GST-GRX480; Lane IV represent expression vector (pKLD66)-GRX480. The arrow indicates induced recombinant protein.

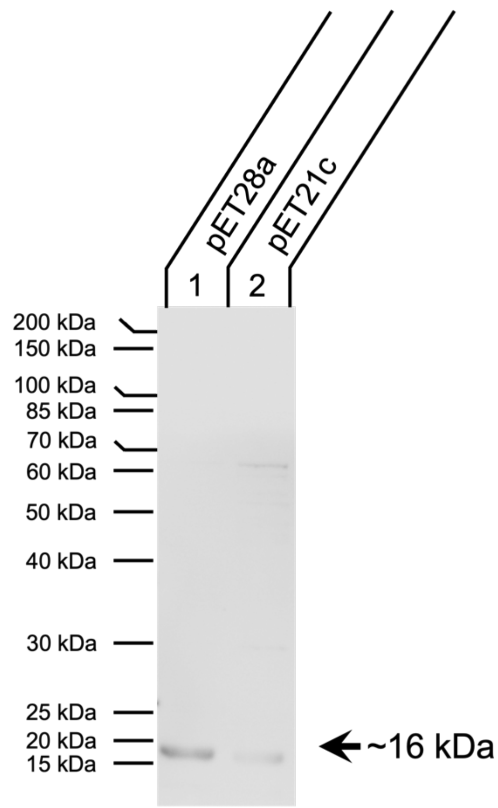

Fig 3. 3: Detection of His tagged- reGRX480 protein

Insitu PB analyses determining the presence of the HIS-reGRX480 protein. For the analysis reducing 13 % SDS-PAGE was employed. PB was analyzed by using the monoclonal anti-HIS antibody. Lane 1: positive control HIS-Tag protein; lane 2: elution fraction of pET21a: reGRX480 system; lane3: elution fraction of pET21c: reGRX480 protein.

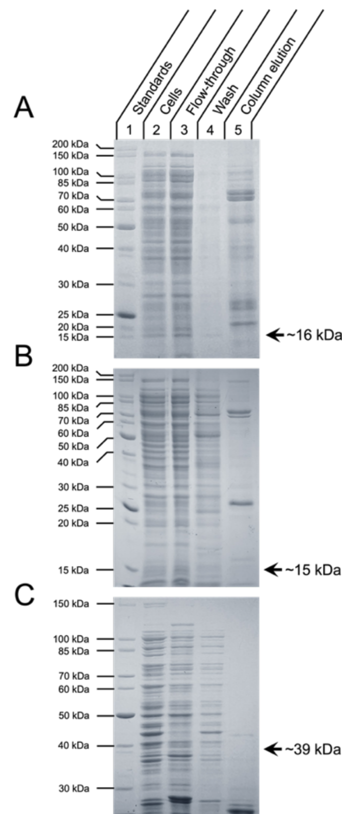

Fig 3. 4: SDS-PAGE analysis of reGRX480 protein using different expression vectors.

For the analysis reducing 13 % SDS-PAGE was employed**. A)** Lane 1: PageRuler™ Unstained Protein Ladder Standards; Lane 2: Total cell extract from E. coli BL21- cells containing pET21a-GRX480; Lane 3: Column flow-through Ni-NTA resin; Lane 4: Column wash fraction; Lane 5: Eluted protein from Ni-NTA column containing with 250mM imidazole. **B)** Lane 1: PageRuler™ Unstained Protein Ladder Standard; Lane 2: Total cell extract from E. coli BL21- cells containing pET21c-GRX480; Lane 3: Column flow-through Ni-NTA resin; Lane 4: Column wash fraction; Lane 5: Eluted protein from Ni-NTA column containing with 125 mM imidazole. **C)** Lane 1: PageRuler™ Unstained Protein Ladder Standard; Lane 2: Total cell extract from E. coli BL21- cells containing pKLD66-GST-GRX480; Lane 3: Column flow-through GSH resin; Lane 4: Column wash fraction; Lane 5: Eluted protein from GSH resin column containing 10 mM GSH. The arrow indicates induced recombinant protein.

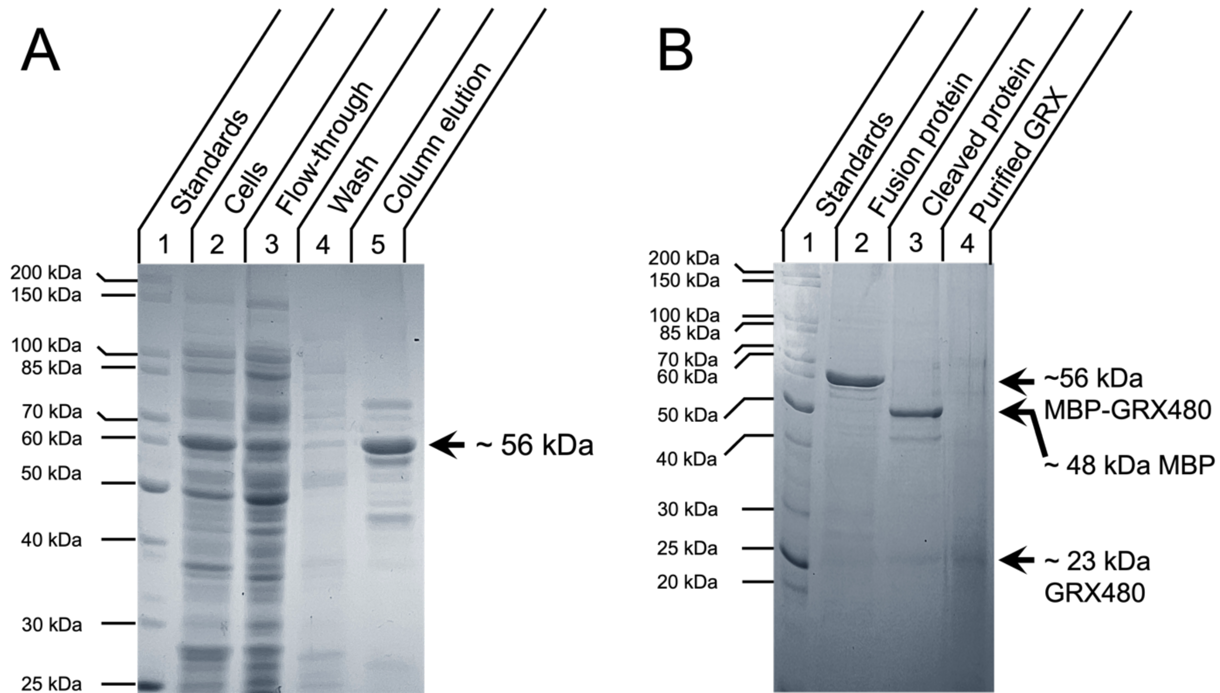

Fig 3. 5 : SDS-PAGE of reGRX480 using pKLD66 expression vector.

For the analysis reducing 13 % SDS-PAGE was employed. **A)** Lane 1: Bio-Rad Precision Plus Protein™ Standards; Lane 2: Total cell extract; Lane 3: Column flow-through Ni-NTA; Lane 4: Column wash fraction; Lane 5: Eluted protein from Ni-NTA column containing with 125 mM imidazole. **B)** Lane 1: Bio-Rad Precision Plus Protein™ Standards; lane 2: His-MBP-reGRX480 protein; lane 3: reGRX480protein after TurboTEV cleavage; lane 4: purified reGRX480 protein. Cleavage of His-MBP-reGRX480 with TurboTEV protease was performed at for 4 hr at 30°C and the cleaved protein was purified by running over a Ni-NTA resin column to remove both the His-MBP tag and His-TEV protease.

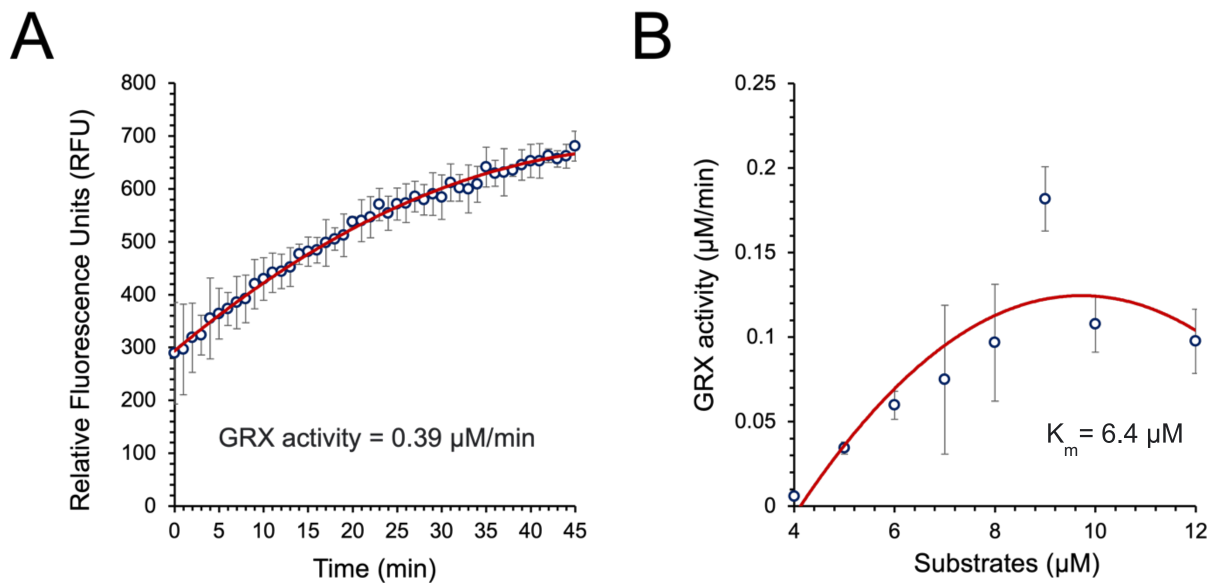

Fig 3. 6: Enzymatic and kinetic analysis of reGRX480

The rate of relative fluorescence units (RFU) and GRX activity were determined using Glutaredoxin fluorometric activity assay kit. The fluorescence emission was recorded at 560 nm after excitation at 520 nm. **A)** Relative Fluorescence Units (RFU) were recorded for 45 minutes, and linear graph was plotted; GRX activity was calculated by following manual instruction. **B)** GRX activity was assayed with various eosin-GS-BSA substrate concentrations in the range of 4-12 μM. Michaelis-Menten plots were used to calculate K_m value.

Tables

Table 3. 1 : A list of Arabidopsis GRXs.

name	locus	location	motif [26]	K_m (mM)	Fe-S**	functions	references
Class I							
GRXC1	at5g63030	cytosol, nucleus	YCGYC	0.08	+	embryo development	[39]
GRXC2	at5g40370	cytosol, nucleus	YCPYC	0.08	−	BS signaling	[39, 40]
GRXC3	at1g77370	ER	YCPYC	0.81	−	floral formation*	[41, 42]
GRXC4	at5g20500	ER	YCPYC	1.13	−		[41]
GRXC5	at4g28730	chloroplast	WCSYC	0.21	+		[43]
Class II							
GRXS12	at2g20270	plastid	WCSYS	+	−		[43]
GRXS14	at3g54900	plastid	MCGFS		+	maintenance of chlorophyll	[44-47]
GRXS15	at3g15660	plastid, mitochondria	QCGFS	+	+	arsenic defense, respiration	
GRXS16	at2g38270	Plastid	QCGFS	+	+	growth	[43, 44, 48]
GRXS17	at4g04950	cytosol, nucleus	CGFS	+	+	auxin signaling in root, flowering, meristem growth tolerance to heat, cold and drought	[46, 49-54]
Class III							
ROXY1	at3g02000	nucleus	TCCMC			petal, and anther development	[4, 55, 56]
ROXY2	at5g14070	nucleus	TCCMC			anther development	[55, 57]
ROXY3	at3g21460	mitochondria*	TCCMS			flower development	[42, 56, 58]
ROXY4	at3g62950	nucleus	SCCMC			GA signaling, flower development	[56]
ROXY5	at2g47870		SCCMC				
ROXY6	at1g06830		SCCLS			nitrogen signaling, homeostasis	[59, 60]
ROXY7	at2g30540		SCCMS				
ROXY8	at3g62960		SCCLC			nitrogen signaling, homeostasis	[59, 60]
ROXY9	at2g47880		SCCLC			· nitrogen signaling, homeostasis	[59-61]

				· Nitrate starvation signaling	
ROXY10	at5g18600		SCCMS		
ROXY11	at4g15700		SCCMS	· CK signaling in root growth, · nitrate-induced root inhibition	[62, 63]
ROXY12	at4g15690	cytosol, nucleus	SCCMS	· CK signaling in root growth, · nitrate-induced root inhibition	[7, 62, 63]
ROXY13	at4g15680		SCCMS	· CK signaling in root growth, · nitrate-induced root inhibition	[62, 63]
ROXY14	at4g15670		SCCMS	· CK signaling in root growth, · nitrate-induced root inhibition	[62, 63]
ROXY15	at4g15660	cytosol, nucleus	SCCMS	· CK signaling in root growth, · nitrate-induced root inhibition	[7, 61, 63]
ROXY16	at1g03020		SCCMS		
ROXY17	at3g62930		SCCMS		
ROXY18	at1g03850	nucleus*	GCCLG	photooxidative stress, floral formation, induced by SA signaling	[57, 64]
ROXY19	at1g28480	nucleus*	GCCMC	SA, JA and OPDA signaling	[12]
ROXY20	at5g11930		SCCMC		
ROXY21	at4g33040		SCCMC		

* Localization prediction; **Fe-S cluster assembly, facilitating electron transfers to target proteins; BS, brassinosteroid; CK, cytokinin; GA, Gibberellin, SA, Salicylic acid; JA, Jasmonic acid, OPDA, Oxophytodienocid acid

Table 3. 2: Oligonucleotide sequence used in this study to clone reGRX480 in respective plasmid.

Plasmids	Directions	Sequence (5′–3′)
pET28a	Forward	ATATTACATATGCAAGGAACGATT
	Reverse	ATATTACTCGAGCAACCACAGAGCCCCAACTTC
pET21c	Forward	TCGATCGGATCCAAATGCAAGGAACGATTTCTTG
	Reverse	TCGATCGTCGACCAACCACAGAGCCCCAACTTC
pKLD66	Forward	ATATTAGGTACCATGCAAGGAACGATTTCTTG
	Reverse	ATATTAAAGCTTTCACAACCACAGAGCCCCAAC
pGEX-4T-1	Forward	TCGATCGGATCCATGCAAGGAACGATTTCTTG
	Reverse	TCGATCGTCGACTCACAACCACAGAGCCCCAAC