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# Protochlorophyllide oxidoreductase protects the oil seed crop plant mustard (*Brassica juncea*) from water-stress

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ABSTRACT

Plant cells lack antioxidative enzyme-mediated reactions for quenching singlet oxygen ($^1O_2$) making it the major cause of damage to plants during daytime. A chlorophyll biosynthesis intermediate protochlorophyllide is a photosensitizer that absorbs light and transfers the energy to $O_2$to generate $^1O_2$.Higher the $^1O_2$ production, greater is the oxidative damage to the plants. Protochlorophyllide oxidoreductase (POR) is a light-dependent enzyme that photo-transforms protochlorophyllide to chlorophyllide using light energy as the substrate. Water-stress severely down regulates the gene and protein expression of POR leading to reduced synthesis of POR enzyme. Therefore, the oil seed crop *Brassica juncea* over expressing the C isoform of POR i.e., PORC along with wildtype (WT) plants were exposed to water stress to ascertain the role of PORC in the protection of plants from drought. The stress treatment was applied to mustard WT and PORC over-expressers (PORCx) plants by withholding water supply up to 8 days. Recovery from stress was monitored up to 48 h after re-watering the drought-treated plants. WT plants wilted after 8$^{th}$ day of drought stress and had lower PSII-dependent electron transport rate (ETR) and initial chlorophyll a fluorescence (Fo)during stress and recovery phase than the PORC over-expressers. Reduced $^1O_2$ produced in *Bj*PORCx plants minimized damage to the photosynthetic machinery allowing for a faster recovery from water stress than the WT plants. Therefore, PORC could be genetically modulated in crop plants to protect them from water-stress.



## 1. Introduction

*Brassica juncea,* an amphidiploid originated due to the natural hybridization between black mustard (*Brassica nigra* (L.) Koch) and turnip mustard (*Brassica rapa* L.) (Szõllõsi, 2020), is a major oilseed crop and is grown in approximately six million hectares in India during the winter season (Singh *et al.*, 2009). With the increase in world population and a steady decline of arable land, enhancing the tolerance of crops to various stress, thereby enabling its maximal productivity is the need of the hour.

Biologically stress for plants is defined as any alteration to the normal physiology, development and functioning that can cause an irreversible damage to the biological machinery. Various environmental factors are responsible for the overall growth and development of plants, any change in these leads to a stress- induced response by the plants.Various abiotic stress, such as drought, flood, extreme temperatures, etc., are serious threats to agricultural practices. Drought spans across continents and has affected several crop plants and the farmers worldwide and it is expected to increase in frequency and intensity due to climate change (Rojas, 2021).

Plant cells have tightly regulated metabolic reactions to minimize production of reactive oxygen species. Under drought stress many metabolic processes including photosynthesis, are negatively affected. Water stress is known to affect thetransfer of electrons from water to NADP by damaging the oxygen evolving complex of PSII (Dalal & Tripathy, 2018). Physiologically, water deficit in plants affects leaf area expansion, absorption of photosynthetically active radiation and subsequently the efficiency of utilization of

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absorbed radiation to carry out carbon fixation in leaves (Flexas *et al.*, 2004). The activity of Rubisco is also impaired by water stress. This ultimately results in complete breakdown of the photosynthetic process, which in turn affects the overall productivity of crop plants(Fariduddin *et al.*, 2009). Several plant species have evolved mechanisms that allow them to adapt and survive periods of water deficit (Cruz de Carvalho, 2008). Mature plants have mechanisms in place to counter reactive oxygen species (ROS) induced damage to the photosynthetic apparatus. ROS can be extremely reactive, singlet oxygen ($^1O_2$) superoxide ($O_2^-$) and the hydroxyl radical ($OH^·$), unlike atmospheric oxygen can oxidize multiple cellular components like proteins and lipids, DNA and RNA. Unrestricted oxidation of the cellular components will ultimately lead to cell death (Tripathy and Chakraborty, 1991; Chakraborty and Tripathy, 1992; Mittler, 2002). To cope with continuous ROS production, plants have numerous enzymatic and nonenzymatic antioxidants, that function as the defense system. The major scavenging mechanisms include superoxide dismutase (SOD), enzymes and metabolites from the ascorbate-glutathione cycle (Foyer-Halliwell-Asada pathway), and catalase (CAT). They are located throughout the different compartments of the plant cell, except for catalase that is exclusively located in peroxisomes (Cruz de Carvalho, 2008).

Chlorophyll (Chl) biosynthetic process yields several tetrapyrrolic intermediates and their degradation products which are photodynamic in nature. These photodynamic molecules absorb light and transfer energy to oxygen molecule that results in production of the ROS i.e., singlet oxygen ($^1O_2$) and subsequent cell death (Chakraborty and Tripathy, 1992; Tripathy *et al.*, 2007). The metabolism of Chl is highly regulated during plant development to prevent ROS production. Previous work in our laboratory on *Arabidopsis thaliana* revealed that overexpression of *AtPORC* resulted in enhanced Chl biosynthesis and conferred tolerance to ALA-mediated oxidative stress (Pattanayak & Tripathy, 2011). The work on overexpressing *AtPORC* gene in *Brassica* genome (Pandey, Tripathy unpublished) have shown similar tolerance to various oxidative stresses (ALA-mediated and salinity). *Arabidopsis thaliana* has three isoforms of POR, namely POR A, POR B, and POR C (Armstrong *et al.*, 1995; Masuda *et al.*, 2003; Oosawa *et al.*, 2000; Pattanayak and Tripathy, 2002; Reinbothe *et al.*, 1996). Among them, POR C is highly expressed in response to light and is found in photosynthesizing tissues (Masuda *et al.*, 2003; Oosawa *et al.*, 2000; Pattanayak and Tripathy, 2002; Vedalankar and Tripathy, 2019). The over expression of POR C enables the conversion of accumulated Pchlideinto Chlide, reducing the possibility of Pchlide derived $^1O_2$ production (Pattanayak& Tripathy, 2011).

POR gene expression and POR protein abundance is severely downregulated in plants leading to impairment of Shibata shift (Dalal and Tripathy, 2012). Therefore, POR C was overexpressed in *Brassica juncea* under the control of a 35S constitutive promoter to ascertain if it could protect plants from water deficit. The impact of drought was monitored on the photosynthetic efficiency of WT and transgenic *Brassica* plants overexpressing POR C (*BjPORCx*) monitoring chlorophyll a fluorescence as its non-invasive signature. It is shown that POR C could protect plants from drought.

## 2. Materials and Methods

*Brassica juncea* cv. VarunaWT and *BjPORCx* (Pandey, Tripathy unpublished data) plants were grown in transgenic greenhouse during the *Brassica* growing season i.e., October to February under a natural photoperiod. The plants were watered at regular intervals and grown till 6 weeks. The watering was then withheld to measure the effect of water stress on various chlorophyll a fluorescence parameters. Measurements were carried out in 2-day interval till 8$^{th}$ day. The plants were then rewatered to study the recovery process of WT and transgenic plants.

Chl a fluorescence measurements were performed in dark-adapted leaves (20 min) (Demmig *et al.*, 1987) at 25°C using portable chlorophyll fluorometer-PAM-2100 (Walz, Effeltrich, Germany); all measurements were repeated 5 times. The minimal (Fo) and maximal (Fm) fluorescence, were measured on leaves that were dark-adapted for 20 min. The instrument uses a low intensity (<0.1µmol photons $m^{-2}$ $s^{-1}$) red measuring beam (650 nm), with a frequency of 0.6KHz for Fo followed by a 0.8 s saturation light pulse of approximately 8,000 µmol photons $m^{-2}$ $s^{-1}$ (20kHz) to measure the maximum fluorescence (Fm). The Fo and the Fm values were used to calculate the PSII quantum yield (Fv/Fm), where Fv is the variable fluorescence (Fv=Fm–Fo).

The light response curves of the electron transport were obtained by measuring fluorescence as a function of increasing actinic light intensity (0 to 1200µmol photons $m^{-2}$ $s^{-1}$). The electron transport rate of photosystem II was calculated from the equation ETR = φPS II x PAR x 0.5 x 0.84, where φPSII is effective PSII quantum yield (calculated by (*Fm' –Ft)/ Fm'=ΔF/ Fm'*) where*Fm'*is referred as the maximum fluorescence yield when the samples are illuminated, and *Ft* is the fluorescence yield at any given time (t). PAR is the photonflux density of incident photosynthetically active radiation, measured in µmol photons $m^{-2}s^{-1}$, 0.5 is the factor of the ratio of PS II and PS

I (1:1), 0.84 is the value thatcorrelates with the percentage of incident photons absorbed by the leaf to drive photosynthesis (Schreiber, 2004).

## 3. Results and Discussion

Overexpression of PORC enabled the plants to recover faster from the water-deficit.

The WT and BjPORCx plants were grown in transgenic greenhouse and water was withheld from 6-week-old plants. Wilting of WT plants was observed on 8th day of water withholding. Chlorophyll a fluorescence parameters were used as a non-invasive signature of photosynthesis (Govindjee, 2004; Schreiber *et al.*, 1995) in control and drought-affected WT and *Bj*PORCx plants. Drought stress primarily causes damage to PSII (White and Critchley, 1999; Dalal and Tripathy, 2012, 2018). PSII actively regulates the electron transport rate and the photochemical efficiency, it prevents or relieves the damage caused by excessive lightenergy to other systems via heat dissipation (Bu *et al.*, 2010). In drought stress, the damage of PSII and antioxidant enzyme system is a non-stomatal limiting factor for the decrease in photosynthetic rate (Li *et al.*, 2017). The energy absorbed by Chl molecule can be used in either one of the three processes, primarily, the energy absorbed by the Chl molecule is directed to initiate the photochemistry; secondly, the absorbed energy could be dissipated in the form of heat; and third, the excited Chl molecules return to the ground state by fluorescence. These are three competing processes. Analysis of modulated chlorophyll fluorescence in response to different light pulses provide valuable information regarding the PSII activity in a quick and non-invasive manner (Blankenship, 2008).

The Fo was determined in dark-adapted leaves of control and water-stressed WT and *Bj*PORCx plants. The Fo of WT and transgenic plants was similar in control conditions. Due to drought treatment the Fo of both plants declined. However, on 8$^{th}$ day of drought, the Fo of WT plants was 14% lower than *BjPORCx* plants. The WT plants substantially wilted on the 8$^{th}$ day of water withholding.Upon rewatering, the Fo of both WT and *Bj*PORCx plants recovered from stress to a large extent (Fig. 1). The Fm decreased both in WT and *Bj*PORCx plants in response to drought. However, on the 8$^{th}$ day the Fm of WT was 15% lower than the transgenics. Upon rewatering the Fm recovered, although it had lower recovery in WT than *Bj*PORCx plants (Fig. 2).

The Fv/Fm declined in WT plants as the stress progressed. The *Bj*PORCx plants always had higher Fv/Fm ratio than that of the WT plants. On day 8 of stress treatment *Bj*PORCx plants had ~20% higher Fv/Fm than water-stressed WT plants. Upon re-watering the *Bj*PORCx plants recovered faster than WT (Fig 3). There was no complete recovery of Fv/Fm from water stress when stress treatment continued

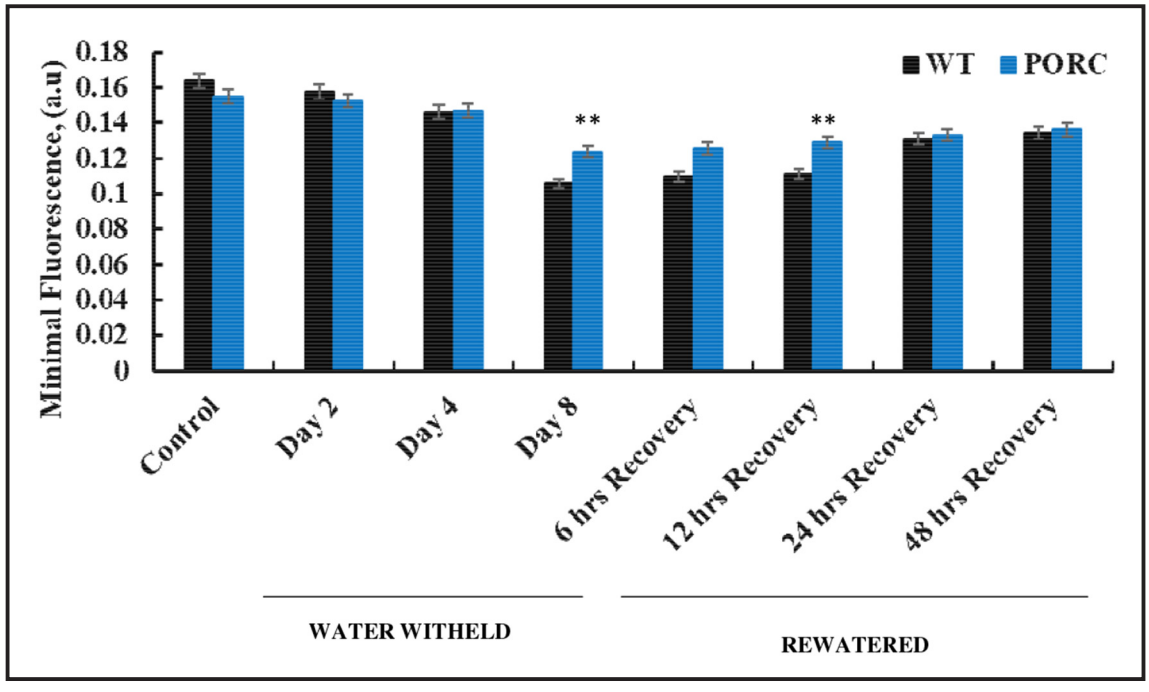


**Fig 1.** Minimal fluorescence (Fo) observed in WT and *BjPORCx* plants after water withholding and rewatering (recovery). Each data point is the average of five replicates, and error bars represent ±SD. Asterisk indicate significant difference determined by *t* test (**P<0.0005).

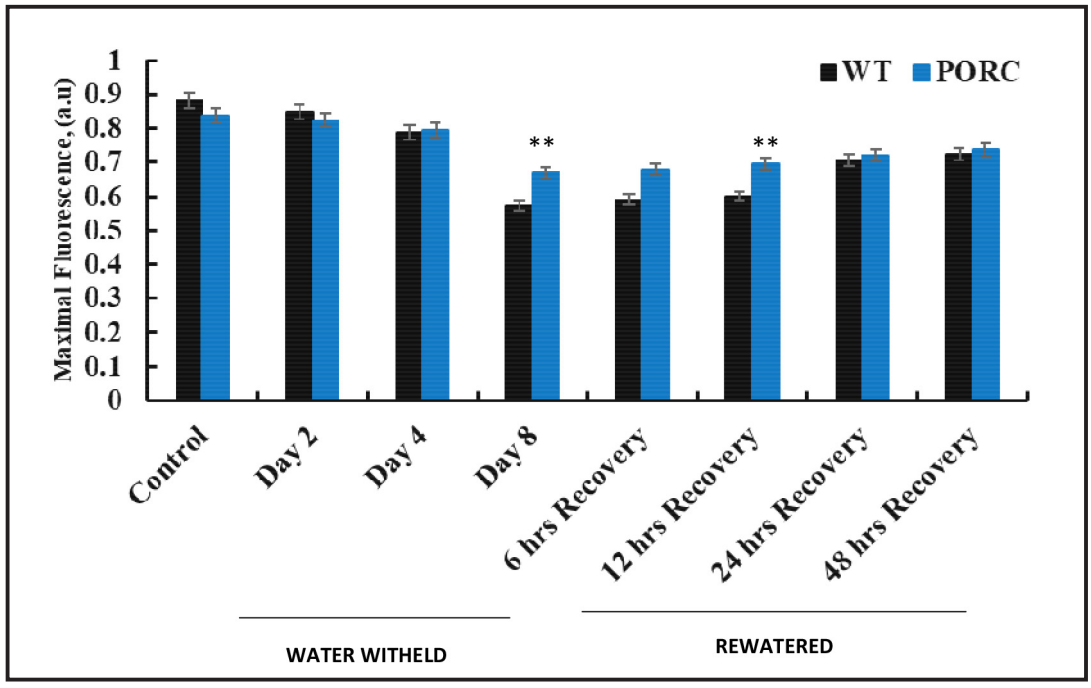


**Fig 2.** Maximal fluorescence (Fm) observed in WT and *BjPORCx* plants after water withholding and rewatering (recovery). Each data point is the average of five replicates, and error bars represent ±SD. Asterisk indicate significant difference determined by *t* test (**P<0.0005).

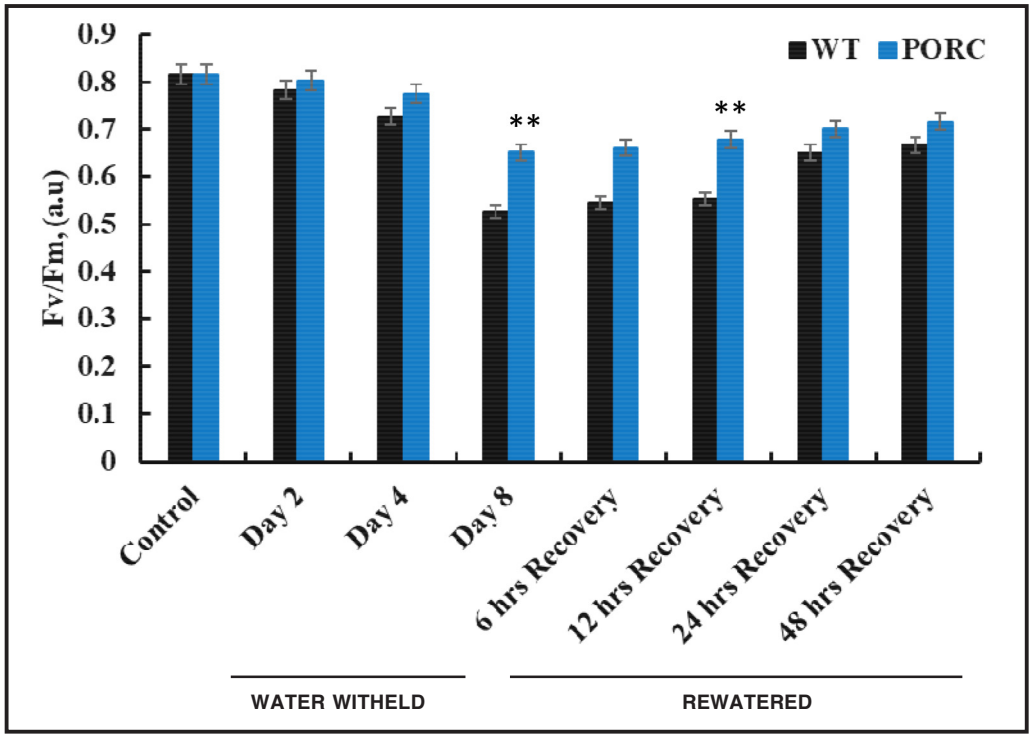


**Fig 3.** Ratio of variable to maximal fluorescence (Fv/Fm) observed in WT and *BjPORCx* plants after water withholding and rewatering (recovery). Each data point is the average of five replicates, and error bars represent ±SD. Asterisk indicate significant difference determined by *t* test (**P<0.0005).

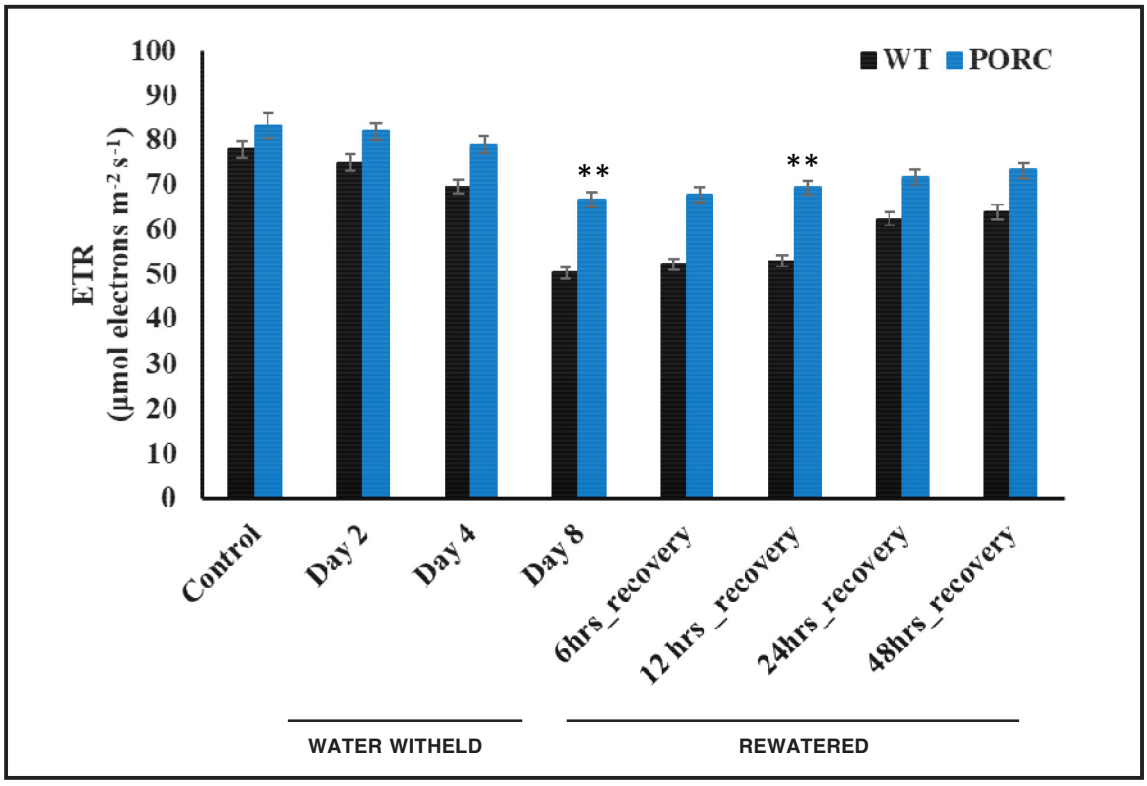


**Fig 4.** Electron transport rate (ETR) of PSII measured in response to photosynthetic active radiation at 1200 µmol photons observed in WT and *BjPORCx* plants after water withholding and rewatering (recovery). Each data point is the average of five replicates, and error bars represent ±SD. Asterisk indicate significant difference determined by *t* test (**P<0.0005).

till 8th day. However, the maximum photochemical efficiency of PSII of *Bj*PORCx plants were always higher than the WT both during stress treatment and recovery phase.

ETR represents the relative photosynthetic electron transport rate expressed as µmol electrons $m^{-2}s^{-1}$, which is calculated based on the measured values of yield of PSII and of PAR (see materials and method). Due to stress treatment, the PSII-dependent ETR decreased both in WT and *Bj*PORCx plants. The ETR of WT plants declined by 36%. Under identical conditions due to 8 days of drought the ETR decreased by 20% in *Bj*PORCx plants. Upon rewatering, although there was not complete recovery, the transgenic plants recovered faster than WT plants, as evident from the ETR data (Fig 4).

Our results demonstrate that *Bj*PORCx plants are more tolerant to drought than the WT. We have shown that Chl biosynthesis is severely impaired due to water-stress. Photo-transformation of Pchlide to Chlide is severely affected by water stress and the Shibata shift is grossly altered (Dalal & Tripathy, 2012). In the PORCx plants, due to generation of abundant POR enzymes, Pchlide is efficiently phototransfomed to Chlide (Pattanayak and Tripathy, 2011). The Chlide produced due to photo transformation is immediately converted to chlorophyll molecules which then associate with the binding proteins present in thylakoid membrane subsequently transferring the absorbed energy to the reaction centers to drive photosynthetic reactions (Pattanayak & Tripathy, 2002, 2011; Tripathy *et al.*, 2004). Non-photo-transformable Pchlide acts as a photosensitizer to generate excess $^1O_2$ in chloroplasts (Ambastha *et al.*, 2020; Chauhan & Tripathy, 2019) and executes programmed cell death in the nucleus by retrograde signaling. The ROS especially $^1O_2$is known for inactivation of PSII and degradation of PSII reaction center D1 protein (Chakraborty and Tripathy, 1991; Graßes *et al.*, 2001a; Trebst *et al.*, 2002; Tripathy and Chakraborty, 1992).

*Bj*PORCx plants with efficient photo-transformation of Pchlide to Chlide due to abundant POR enzyme resulting in reduced accumulation of the photosensitizer Pchlide generated minimal reactive oxygen species (ROS) more specifically $^1O_2$. This resulted in smaller damage to PSII and its faster recovery upon re-hydration than the WT plants..

## 4. Conclusion

POR expression and activity is substantially decreased due to Water stress. This causes Pchlide accumulation and $^1O_2$ generation in light. Therefore, it is important to overexpress PORC to photo-transform accumulated Pchlide

to Chlide to minimize the accumulation of non-phototransformable Pchlide generated during stress condition. Due to efficient photo-conversion of Chl biosynthesis intermediate Pchlide by the abundant POR enzyme to Chlide non-photo transformable Pchlide does not accumulate, and this results in reduced generation of $^1O_2$ and consequently smaller damage to the photosynthetic apparatus.

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