Chlorophyll is the most abundant pigment on earth responsible for absorption of sunlight for photosynthesis. Light absorbing properties of Chl make it a potential cellular phototoxin. Chl biosynthetic intermediates and its degradation products are photodynamic in nature. These photodynamic molecules absorb light and transfer energy to oxygen molecule that results in production of ROS and cell death. The metabolism of Chl is highly regulated during plant development to prevent ROS production.

During senescence and fruit ripening, Chl a after Mg-dechelation is hydrolyzed in to free phytol and pheophorbide in Arabidopsis (Pruz'inska' et al., 2005) whereas it is degraded to Chlide and phytol in Citrus (Jakob et al., 1999). Chlide is further degraded in to primary fluorescent Chl catabolites (pFCCs) and nonfluorescent Chl catabolites NCCs (Pruz'inska' et al., 2003; Gray et al., 2004). However, phytol produced during Chl degradation is usually recycled for Chl resynthesis using Chlide as a substrate by the enzyme Chl synthase (Chl G). This recycling conserves several energy resources including NADPH. The phytol before esterification with Chlide is phosphorylated by phytol kinase (VTE5) to phytyl phosphate (Ischebeck et al., 2006 and Valentin et al., 2006). The latter is further phosphorylated to Phytyl diphosphate by an yet unidentified kinase. The phytyl diphosphate could also be metabolized to α-tocopherol via homogentisate phytyltransferase (VTE2), 2-methyl-6-phytylbenzoquinol methyltransferase (VTE3), tocopherol cyclase (VTE1) and γ-tocopherol methyltransferase (VTE4) enzymes. The tocopherols are efficient quenchers of $1^2O_2$. As tocopherols are highly hydrophobic they are located in the membrane bilayer and protect the biomembranes from $1^2O_2$-induced injury.

Our results clearly demonstrate that genetic manipulation of Chl and tocopherol biosynthesis in Arabidopsis and Brassica via overexpression of Phytol kinase (VTE5) augments the amount of Chl and tocopherol (vitamin E). Increased Chl synthesis coupled with tocopherol mediated quenching of $1^2O_2$ and other reactive oxygen species leads to increased photosynthesis, plant productivity and grain yield in normal growth conditions as well as in a stressful environment.

**Genetic manipulation of Vitamin E pathway gene 5 (VTE5) in Arabidopsis**

Vitamin E pathway gene 5 (VTE5) encodes phytol kinase which is responsible for reutilization and activation of phytol, generated during Chl catabolism. Bioinformatic analysis of deduced amino acid sequence of AtVTE5 reveals that it is a chloroplast targeting enzyme having phosphatidate cytidylyltransferase activity and its homologous sequences are also present in archaea, eubacteria, cyanobacteria and plants (Valentin et al., 2006).
Hydropathy plot shows that \textit{AtVTE5} protein has 6 transmembrane helices indicating to be a transmembrane protein (Fig. 1). Antigenecity plot of \textit{AtVTE5} shows its antigenicity towards the binding of monoclonal antibody. Only one stretches of hydrophilic amino acids of \textit{AtVTE5} determine the probable epitope region (Fig. 2).

Chl metabolism is developmentally regulated process. Study of tissue specific expression of \textit{AtVTE5} reveals its maximal expression in old leaves which are in senescence stage having increased Chl degradation (Fig. 3). \textit{VTE5} expression was minimal in 1\textsuperscript{st} week and increased after 7\textsuperscript{th} week (Fig. 4). These expression studies clearly demonstrate that \textit{AtVTE5} expression is developmentally regulated and its expression increases during senescence when Chl degradation is in progress.

As compared to that of WT, the gene expression and the protein abundance of phytol kinase significantly increased in \textit{AtVTE5x} plants and declined in \textit{antiVTE5} plants (Fig. 11-13). \textit{AtVTE5x} plants were bigger in size, flowered earlier and had longer root than that of WT plants whereas \textit{antiVTE5} plants had similar phenotypic growth as compared to that of WT (Fig. 14-16). Fresh weight, dry weight and floral diameter were also higher in \textit{AtVTE5x} plants compared to WT and \textit{antiVTE5} plants (Fig. 17).

The Chl contents of \textit{AtVTE5x} plants were higher than that of WT. However, in \textit{antiVTE5} plants Chl contents were lower than that of WT (Fig. 18). Increased Chl synthesis in \textit{AtVTE5x} plants may be due to increased esterification of Chlide, a photodynamic singlet oxygen generating compound, and phytol to Chl. Decreased production of phytyldiphosphate, an intermediate for Chl biosynthesis in antisense plants led to downregulation of Chl biosynthesis.

Increased production of phytylphosphate in transgenic plants resulted in huge i.e., 300\% increase in total tocopherol contents in sense plants (Fig. 19-20). All the measured components of different isoforms of tocopherol and especially that of most active \textit{a}-tocopherol increased in the \textit{AtVTE5x} plants. In opposite vein, the downregulation of \textit{AtVTE5} expression led to reduced tocopherol contents in antisense plants.

Tocopherol content usually increases in stress-full growth conditions. Therefore, increased production of tocopherol in sense plants should protect them from stress. Increased synthesis of tocopherols especially that of most active \textit{a}-tocopherol protected plants from salt-stress. \textit{AtVTE5x} plants had higher (10-50\%) of seed germination and \textit{antiVTE5} plants had lower (6-25\%) seed germination than that of WT plants in the presence of 100-200 mM NaCl (Fig. 21-23). Similarly the increased tocopherol contents in sense plants alleviated the delay
in germination of *Arabidopsis* in 200 mM salt-stress. It is due to increased or decreased tocopherol content in sense and antisense plants respectively.

A primary function of tocopherols in plants is to limit nonenzymatic lipid oxidation during seed storage, germination, and early seedling development (Sattler et al. 2004). Seed germination assay and seed viability assay under stress condition clearly demonstrate the tolerance of *AtVTE5* overexpression line and sensitivity of antisense line to salt stress as evident from higher Chl content and better growth in sense plant and lower Chl content and reduced growth in antisense plants compared to WT plants (Fig. 24-27).

Due to increased Chl and tocopherol content photosynthetic efficiency of *AtVTE5x* plants was higher than that of WT plants. Conversely, due to decrease Chl and tocopherol content in *antiVTE5* there photosynthetic efficiency declined. As shown in Figure 28A, the Fo declined in 100 mM NaCl-treated WT and antisense plant this was probably due to decline of Chl contents. In 150 mM NaCl stress Fo was substantially increased in WT plants. This could be due to several reasons inactivation of PSII (e.g. due to formation of non-QB centers) and separation of light-harvesting complex II from PSII (Schreiber and Armond, 1978; Bilger et al., 1984, 1987; Ducruet and Lemoine, 1985; Bukhov et al., 1990; Cao and Govindjee, 1990; Havaux, 1993; Yamane et al., 1997). The decrease in Fm of antisense plants grown in 100 mM and 150 mM NaCl could be due to decrease in quantum yield of PSII (Fig. 25B). The Fv/Fm ratio, a measure of the quantum efficiency of dark-adapted leaves, declined in WT and antisense plants grown in 100 mM and 150 mM NaCl (Fig. 25C). However, at all NaCl levels the sense plants always had higher Fv/Fm ratio and antisense plants had a lower ratio than that of WT. This was probably due to increased tocopherol content in sense plants resulting in reduced accumulation of reactive oxygen species (ROS), generated during salt stress. This could protect the D1 protein from accelerated degradation and maintain PSII active under stress condition. In antisense plant due to lower tocopherol production, ROS accumulation would have increased, resulting higher D1 degradation and reduced electron transport. In fact the estimated ETR was higher in sense and lower in antisense plants especially in salt-stress condition. The ETR (μmoles electrons m⁻² s⁻¹) increased in response to photosynthetic active radiation (PAR) (μmoles photons m⁻² s⁻¹) (Fig. 29). Due to 100-150 mM NaCl-stress, the ETR decreased both in low light (10-30 μmoles photons m⁻² s⁻¹) and higher light intensities (100-300 μmoles photons m⁻² s⁻¹) in WT and to a larger extent in antisense plants. This suggests a damage to the reaction center of PSII rather than degradation or detachment of LHCPs. Under identical conditions the stress-induced
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downregulation of ETR was considerably lower than that of WT in *AtVTE-5x* plants most likely due to tocopherol-mediated protection of plants from ROS.

The qP is a measure of the fraction of still open PSII reaction centers. The same decreased in response to photosynthetic active radiations (PAR) (μmoles photons m⁻² s⁻¹). In sense plants the qP was higher than that of WT while in antisense plants the same was lower in all NaCl level (Fig. 31). The qN is a measure of heat dissipation and a combined total for the combination of photo-protective mechanisms, state 1 and state 2 transition quenching, and photo-inhibition and photo-damage. The qN increased in response to higher light intensities. In sense plants the qN was lower than that of WT, while in antisense plants the same was higher in control as well as in 150 mM NaCl (Fig. 32). These observations clearly demonstrate that because of higher Chl and tocopherol content in sense plants, they had more active PSII, resulting better photosynthetic efficiency under normal as well as in stress condition than that of WT and antisense plants.

**Overexpression of *AtVTE5* in *Brassica juncea***

Our study in model plant *Arabidopsis thaliana* revealed that overexpression of *AtVTE5* could protect plants from deleterious effect of salt stress. To ascertain if the same could protect a crop plant, *AtVTE5* was overexpressed in *Brassica juncea* plants under the control of constitutive *Cauliflower mosaic virus* (CaMV) 35S promoter with omega enhancer (Fig. 33-35). The integration of *AtVTE5* gene in *Brassica* genome was confirmed by Southern blot analysis. Figure 36 showed the single integration of transgene in VTE5x-2 and VTE5x-3 line and twice integration of transgene in VTE5x-1 line. Semi-quantitative RT-PCR analysis showed the higher *AtVTE5* transcript in transgenic plants (Fig. 37). VTE5 protein was also significantly higher in transgenic plants than that of WT as evident from Western blot analysis (Fig. 38).

VTE5 is a key enzyme responsible for recycling of phytol generated from Chl degradation reaction for the synthesis of new Chl molecules via esterification of Chlide with phytanyl diphosphate. As in *Arabidopsis*, the overexpression of *AtVTE5* in *Brassica* resulted in increased Chl contents (Fig. 39) due to higher mobilization of phytol for esterification with newly synthesized Chlide. The phosphorylation product of phytol kinase (VTE5) is further phosphorylated and serves as a remobilized substrate for chlorophyll synthase.

The tocopherols are one of the most important antioxidants present in the cell and are involved in the quenching and scavenging of various reactive oxygen species and act as a recyclable chain reaction terminator of poly unsaturated fatty acid free radicals generated by
lipid peroxidation (Freyer, 1992; Kamal-Eldin & Appelquist, 1996; Bramley et al., 2000; Fukuzawa & Gebicky, 1983; Neely et al., 1988; Tappel, 1962; Burton & Ingold, 1986; Esterbauer, 1991). The total tocopherol and especially that of most active α-tocopherol increased in VTE5x Brassica plants due to augmented mobilization of phytol produced from isoprene metabolic pathway as well as from Chl degradation for tocopherol synthesis (Fig 40, 41). Increase in tocopherol content protected the leaf discs from salt-stress. The leaf discs from the transgenic plants showed significant tolerance against high concentrations of NaCl as compared to that of WT. This was evident by delayed bleaching and senescence and presence of higher Chl content in the leaf disc of transgenic plants as compared to that of WT plants (Fig. 42).

Vermiculite-grown one month old VTE5x plant treated with salt for 8 weeks showed tolerance. In WT plants the flowering was substantially delayed. The transgenic plants had better growth than that of WT plants (Fig. 43). Chl and carotenoid contents, measured after 4 weeks of salt treatment, were higher in VTE5x plants than those of WT (Fig. 44). The chloroplast ultrastructure of transgenic plants revealed tolerance to salt stress as evident from reduced structural distortion of thylakoids in transgenic plants (Fig. 45). This clearly demonstrates that tocopherol, a lipophilic antioxidant associated to biomembranes, protect cell membrane from salt-stress-induced oxidative stress. This is evident from reduced production of $^{1}$O$_2$ (Fig. 51) in the transgenic plants due to its efficient quenching by increased amounts of the most bioactive α-tocopherol in the membranes. Fo, Fm and Fv/Fm of WT plants were decreased in salt stress (Fig. 46). However, the transgenic plants always had higher Fv/Fm ratio in stress condition than that of WT. There was no change in Fo and Fm of transgenic plants under same growth conditions. This was probably due to increased tocopherol content in transgenic plants resulting in reduced accumulation of reactive oxygen species (ROS), generated during salt stress. Increased tocopherol content is known to protect PSII reaction center D1 protein from oxidative stress (Trebst et al., 2002; Grabes et al., 2001).

In high light, absorption of photons by leaves increases almost linearly. However, the rate of photosynthesis reaches the maximum value much before the linear increase in light absorption ceases. Therefore the plants end up in absorbing more light than they could utilize in photosynthesis. This results in the over excitation of the photosynthetic apparatus. In the presence of excess light energy, the QA and Q$_B$ (the first and second plastoquinone electron acceptors of Photosystem II, PS II) in the electron transport chain are over reduced (Barber and Andersson, 1992) and because of that, charge separation cannot be completed between P680 and pheophytin and the triplet state of the reaction center Chl P680 ($^{3}$P680) is favored.
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(Aro et al., 1993; Ohad et al., 1994) leading to the formation of $^1\text{O}_2$ (Foote et al., 1984). Normally when excess light is absorbed, an alternative dissipating pathway is activated that safely returns $^1\text{Chl}^*$ to its ground state before it can convert to $^3\text{Chl}^*$. The excitation energy of excess $^1\text{Chl}^*$ is dissipated by carotenoids in Chl and carotenoid binding protein complexes (Baroli and Niyogi, 2000; Baroli et al., 2003, 2004; Davison et al., 2002; Pogson and Rissler, 2000). The carotenoids, which quench the excited state of Chl, must be in close proximity with triplet Chl i.e., within maximum distance of 3.6 Å. In this spin exchange reaction, the triplet state of carotenoid is formed that can dissipate the excess energy as heat. In the reaction center, distance between Chl and carotenoid is too large to allow triplet quenching. Therefore $^1\text{O}_2$, produced in the reaction center can not be easily dissipated by carotenoids. The release of $^1\text{O}_2$ was detected in thylakoids (Chakraborty and Tripathy, 1992; Fryer et al., 2002, Hideg et al., 1998). Generation of $^1\text{O}_2$ leads to accelerated degradation of PSII reaction center D1 protein. The presence of higher amount of tocopherol in the thylakoid membrane in $VTE5x$ plants could quench $^1\text{O}_2$ produced from the reaction center and prevent D1 degradation resulting in protection of plants from oxidative stress. Presence of well-developed grana and stroma thylakoids in transgenic plants and higher Chl content resulted higher photosynthetic efficiency of the transgenic lines i.e., electron transport rate (Fig. 47), quantum yield of PSII (Fig. 48), photochemical quenching of Chl a fluorescence (Fig. 49) than that of WT plants after NaCl treatment. As generation of $^1\text{O}_2$ was lower in transgenic plants in stress condition (Fig. 51), their lipid peroxidation was lower resulting in reduced MDA production (Fig. 52). As damage to cell membrane was smaller in transgenic plants, they had reduced electrolyte leakage (Fig. 53).

$VTE5x$ plants of Brassica exhibited early flowering and fruit setting. Salt stress (150 mM, EC ~17 dSm-1) was applied to soil-grown potted WT and transgenic plants grown in green house revealed that $VTE5x$ plants were tolerant to salt stress. After 4 weeks of stress the flowering was delayed by two weeks in WT plants. Under identical conditions the $VTE5x$ plants flowered. After 10 weeks of application of salt, both WT and transgenic had fruits although the fruiting was delayed in WT plants and they had reduced number of seeds per plant (Fig. 56). This is due to presence of higher amounts of Chl and tocopherols in transgenic plants.

Overexpression of $AtVTE5$ in Brassica modulated photosynthesis and plant productivity. Net photosynthetic rate of transgenic plants was higher in control as well as in stress condition (Fig. 55). Their quantum yield of CO$_2$ assimilation increased from 0.069 in WT to 0.076 in $VTE5x$ plants. The relative quantum yield of 0.069-0.074 for CO$_2$
DISCUSSION

assimilation is similar to that observed in several C3 plant species (Ehleringer and Pearcy, 1983). The light-saturated net CO₂ assimilation rate was higher in VTE5x plants in normal growth conditions that matches with that of ETR data. Increased rate of respiration in the transgenic plants enhance the light compensation point and contributed to augmented crop growth. Due to salt treatment the light saturated photosynthesis rate declined by 10% whereas in VTE5x plants it was not significantly affected. The quantum yield of photosynthesis in WT and VTE5x plants was not affected due to salt stress.

Higher rate of photosynthesis in normal growth conditions in VTE5x plants resulted in increased plant productivity and grain yield by 21% (Fig. 56). In salt stress condition VTE5x plants had higher grain yield, more number of seeds per plant and higher grain yield than that of WT. Due to longer flowering period and longer grain filling duration the unit seeds weight was higher in transgenic plants in control as well as in stress conditions.

These results demonstrate that the quenching of ROS, specifically that of ¹O₂ by membrane bound lipophyllic compound α-tocopherol could protect plant from oxidative stress and contribute increased plant productivity and grain yield (21%). This was more evident in VTE5x plants grown in salt environment where plant productivity, grain filling and grain yield were much higher than that of WT. However these transgenic plants need to be tested in actual field condition for the response to salt stress-induced oxidative stress.

Overexpression of AtPORC in Brassica juncea

Chloroplast is the site of photosynthesis, having a highly organized thylakoid membrane system that harbours all components of the light-capturing photosynthetic apparatus and has appropriate structural organization for optimal light harvesting. Chl is the main light absorbing pigment and is present both in the light harvesting complex (LHC) and the photosynthetic reaction centers. Inefficient transfer of energy results in the generation of triplet state Chl that reacts with triplet oxygen to produce the highly reactive ¹O₂ by type II photosensitization reaction. Not only Chl but its biosynthetic intermediates produce ¹O₂ in the presence of light. Oxygen which is produced during photosynthesis can also generate other reactive oxygen species (ROS) besides ¹O₂ by type I photosensitization reaction. During steady state ROS are scavenged by various enzymatic and nonenzymatic antioxidative defence mechanisms. In a normal cell there is an equilibrium between ROS production and their detoxification. Under stress condition i.e., salinity, drought, heavy metals, herbicides, high temperature and chill, this equilibrium is perturbed. These disturbances in equilibrium leads to sudden increase in ROS level resulting cell damage and death.
Previous work in our laboratory on *Arabidopsis* revealed that overexpression of *AtPORC* resulted enhanced Chl biosynthesis. Its overexpression in *Arabidopsis* plants conferred tolerance to ALA-mediated severe oxidative stress. Overexpression of *At PORC* in *Arabidopsis* plant resulted efficient photo-transformation of Pchlide to Chlide that decreases Pchlide contents and consequently releases the Pchlide-induced feed-back inhibition of ALA biosynthesis. Increased synthesis of ALA in *PORCx* plants upregulated the gene/protein expression of the downstream Chl biosynthetic enzymes that elucidates a regulatory net work of gene expression of tetrapyrrole biosynthetic enzymes. The coordinated upregulation of gene/protein expression of several enzymes involved in tetrapyrrole biosynthesis leads to enhanced Chl accumulation, larger LHC II and greener phenotype. Furthermore, due to increased photo-transformation of Pchlide and enhanced rate of Chl synthesis, Pchlide and other intermediates of Chl biosynthesis decreased and consequently, generation of $^1O_2$ was reduced. Therefore, the $^1O_2$-mediated photo-oxidative damage in high-light-stressed *AtPORCx* plants was minimal. This work clearly demonstrates that photo-oxidative damage and ultimate plant death caused by ALA could be substantially minimized by overexpression of PORC that limits the generation of $^1O_2$.

In present study, PORC was overexpressed in *Brassica juncea* under the control of constitutive *CaMV* 35S promoter with omega enhancer. Southern blot analysis confirmed the single integration of *AtPORC* gene in *Brassica* genome (Fig. 60). Increased accumulation of *AtPORC* transcripts (Fig. 61, 62), and PORC protein (Fig. 63) in *Brassica PORCx* plants, resulted in higher (19-23%) production of Chl (Fig. 64). As in *PORCx Arabidopsis* plants, the *Brassica PORCx* plants may have coordinated upregulation of gene/protein expression of several enzymes involved in tetrapyrrole biosynthesis leading to enhanced Chl accumulation and greener phenotype.

In plants there is a strong regulation of Chl biosynthesis leads to optimal accumulation of Chl and heme biosynthetic intermediates. Because these intermediated are photodynamic in nature and endogenously generate reactive oxygen species after absorption of light. Triple excited forms of tetrapyrroles are produced in light that interact with molecular oxygen ($O_2$) and generate $^1O_2$ via type II photosensitization reaction. Application of 5-aminolevulinic acid (ALA) results in the overaccumulation of protochlorophyllide (Pchlide) in dark (Granik, 1959; Tripathy and Rebeiz, 1985, 1986; Chakraborty and Tripathy, 1990, 1992a, 1992b; Tripathy and Chakraborty, 1991). Pchlide is a feedback inhibitor of ALA synthesis (Stobart and Ameen-Bukhari, 1984, Richter et al., 2010) that inhibits the glutamyl-tRNA reductase (GluTR) enzyme. Application of ALA at sun set bypasses the
feedback inhibition site (GluTR) resulting in overnight overaccumulation of Chl biosynthetic intermediates i.e., ProtoIX, MgProto and Pchlide. After the day break, these overaccumulated tetrapyrroles specifically Pchlide absorbs light and goes to excited state. Upon return to ground state the excess absorb energy can not be transferred to photosynthetic reaction center as Chl biosynthetic intermediates are not integral part of the light harvesting antenna. In state the excited Pchlide molecules transfer their energy to triplet oxygen molecule to generate highly reactive $^1O_2$. Later is highly reactive compound and attacks the electron dense poly unsaturated fatty acids to produce lipid peroxides leading to damage of thylakoid ultrastructure and injury to plasma membrane that causes ultimate plant death.

To study the real effectiveness of PORC overexpression to minimize the $^1O_2$ generation in light, our previous approach to over-produce Pchlide by spraying the plants with ALA (Tripathy and Chakraborty 1991) that bypasses the tetrapyrrole-mediated regulatory feedback inhibition, was probed further.

Exposure of ALA-treated dark incubated (14 h) WT Brassica plants to light caused wilting of leaves followed by appearance of prominent necrotic patches and ultimate plant death (Fig. 65, 66). This was due to excess accumulation of photosensitizer Pchlide. Similar effects were also reported previously in ALA-treated Arabidopsis, cucumber and other plants (Tripathy and Chakraborty, 1991; Chakraborty and Tripathy, 1992; Rebeiz et al., 1984). Application of diphenylether (DPE) herbicides including nitrofen, oxyfluorfen, acifluorfen on plants leads to overaccumulation of Proto IX in light that results in generation of $^1O_2$ and severe oxidative stress (Kunert and Boger, 1981; Orr and Hess, 1981; Lambert et al., 1984, Gupta and Tripathy, 1999, 2000; Lermontova and Grimm, 2000, Tripathy et al., 2007). Deregulation of Chl biosynthetic machinery by antisense expression of some of the biosynthetic pathway genes i.e., suppression of the message abundance of UroD in tobacco (Mock and Grimm, 1997; Mock et al., 1999) and in maize (Hu et al., 1998), CPX in tobacco (Kruse et al., 1995a, b), maize (Williams et al., 2006) and Arabidopsis (Ishikawa et al., 2001), PPX 1 in Arabidopsis (Molina et al., 1999), ferrochelatase in tobacco (Papenbrock et al., 2001) leads to the accumulation of different biosynthetic intermediates leading to light-induced oxidative damage.

Continued light exposure of ALA-treated and dark-incubated Brassica plants led to the death of WT plants within 48 h (Fig. 65). In contrast to WT plants, ALA-treated Brassica PORCx plants incubated in dark (14 h) and subsequently exposed to light (50 μmoles photons m$^{-2}$ s$^{-1}$) had minimal damage to their leaves (Fig. 65). The Brassica PORCx plants survived even after 7 d of continuous light (50 μmoles photons m$^{-2}$ s$^{-1}$) exposure. 2 mM and 3 mM
CONCENTRATIONS of ALA did not result in the substantial damage to *Brassica PORCx* plants whereas WT plants perished after 7 days of light exposure (Fig. 65F, G). For phototransformation of Pchlide to Chlide, it is prerequisite for Pchlide to bind to POR and NADPH to form a multimeric ternary complex. Without binding of Pchlide with POR and NADPH, phototransformation of Pchlide to Chlide can not occur. The tolerance of *Brassica PORCx* plants to ALA treatment was due to the abundant amount of PORC protein that efficiently photoconverted excess Pchlide to Chlide after 20 min of light exposure (Fig. 70) leading to reduced accumulation of photosensitizer Pchlide in light than that of WT plants. Reduced accumulation of Pchlide in the *Brassica PORCx* plants led to decreased production of $^{1}O_{2}$ (Fig. 71) and minimal cell damage (Fig. 72, 73) and no plant death.

The Chl a fluorescence was used as a non-invasive tool to monitor cell membrane damage (for reviews see Govindjee, 1995, 2005). The Fo emanating from a dark-adapted leaf is the minimal fluorescence when all the reaction centers are open and the Fm is the maximal fluorescence when all the reaction centers are closed. The Fv is the variable fluorescence, which represents Fm-Fo (Govindjee, 1995). The Fv/Fm indicates the maximum quantum efficiency of photochemical reaction of PSII. The Fv/Fm (Fig. 68), ETR (Fig. 69A), ΦPSII (Fig. 69B) and qP (Fig. 69C) of Chl a fluorescence declined in ALA-treated WT plants exposed to light for 1 h. Continued light exposure up to 2-6 h resulted in substantial decrease of the above parameters (Fig. 68 and 69A,B,C). Higher production of $^{1}O_{2}$ in ALA-treated WT plants damages photosynthetic apparatus and therefore could be a reason for substantial reduction in photosynthetic efficiency of ALA treated WT plants after light exposure.

Peroxidation of membrane lipids is one of the phytotoxic consequences of oxidative stress (Kenyon and Duke, 1985; Duke and Kenyon, 1986; Tripathy and Chakraborty 1991; Gupta and Tripathy, 2000). Lipid peroxidation is a complex process where $^{1}O_{2}$ reacts directly with the electron dense polyunsaturated membrane lipids to form semi stable hydroperoxides (Pryor, 1976). MDA production is considered as an index of lipid peroxidation. ALA induced photodynamic reactions caused membrane lipid peroxidation that resulted in accumulation of increased amounts of MDA in leaf tissues of WT plants (Fig. 72). In *BjPORCx* plants due to reduced lipid peroxidation smaller amounts of MDA was produced (Fig. 72). Although the death and complete photobleaching of the WT plants occurred after 24 h of light exposure, the cell membrane was substantially damaged even after 2 h of light exposure as revealed by electrolyte leakage (Fig. 73). In *BjPORCx* plants the damage to cell membrane was minimal (Fig. 73).
DISCUSSION

Our results demonstrate that ALA could be used as selective commercial herbicide. ALA could be produced from levulinic acid by addition of amino group at C5 position (Bozell et al., 2000). The precursor of ALA, i.e., levulinic acid could be produced cheaply from cellulosic material of organic waste in a chemical reactor (Hayes et al., 2005). Biotechnologically, ALA (20 mM) could also be produced cheaply by extra-cellular secretion of E.coli overexpressing ALA synthase i.e., hemA from Bradyrhizobium japonicum expressed under the control of T7 promoter (Choi et al., 1999).

It is concluded that overexpression of PORC results in efficient photo-transformation of Pchlide to Chlde that decreases Pchlide contents and consequently releases the Pchlide-induced feed-back inhibition of ALA biosynthesis. Furthermore, due to increased photo-transformation of Pchlide and enhanced rate of Chl synthesis, Pchlide accumulation decreased and consequently, generation of $^{1}\text{O}_{2}$ was reduced. Therefore, the $^{1}\text{O}_{2}$-mediated photo-oxidative damage in ALA-treated Brassica PORCx plants was minimal. It was earlier shown that ALA application to plants could cause photo-oxidative damage and ultimately kill plants. The present study demonstrates that photo-oxidative damage and ultimate plant death caused by ALA could be substantially minimized by overexpression of PORC that limits the generation of $^{1}\text{O}_{2}$. This approach could be biotechnologically exploited further to use ALA as a commercial selective photodynamic herbicide.

Overexpression of AtPORC in Brassica confer tolerance to salt stress

To study the tolerance of Brassica PORCx plants to salt stress, the leaf discs of WT and Brassica PORCx plants were subjected to salt tolerance test. The leaf discs from the transgenic plants showed significant tolerance against high concentrations of NaCl as compared to that of WT. This was evident by delayed bleaching and higher Chl content in the leaf disc of Brassica PORCx plants as compared to that of WT (Fig. 74).

Vermiculite-grown one month old Brassica PORCx plant irrigated with half-strength Hoagland solution+NaCl for 8 weeks showed significant tolerance. The transgenic plants had better growth than that of WT plants (Fig. 75). The continued irrigation of WT plants with half-strength Hoagland solution along with 100 mM or 150 mM NaCl perished; 200 mM NaCl caused plant death faster. Under identical conditions, Brassica PORCx plants survived, flowered and had pods although their growth, development and fruit setting was substantially downregulated as compared to untreated controls (Fig. 76). Chl and carotenoid contents, measured after 4 weeks of salt treatment, were higher in Brassica PORCx plants than those of WT (Fig. 76). Reduced structural distortion of thylakoids i.e., lack of swollen thylakoids and
near-intact granal organization in *Brassica PORCx* plants revealed their tolerance to salt stress (Fig. 77).

To understand the mechanism of tolerance of *Brassica PORCx* plants to salt stress their Chl biosynthetic product Pchlide and Chl degradation product Pheophorbide a were estimated. PORC is involved in phototransformation of Pchlide to Chlide. Overaccumulation of POR results in efficient phototransformation of Pchlide to Chlide leading to reduced accumulation of photosensitizer Pchlide in *Brassica PORCx* plants. In response to salt the PORC activity decreases (Satpal and Tripathy unpublished). Therefore, in response to salt WT plants accumulated higher amounts of Pchlide than that of untreated controls. As PORC is constitutively overexpressed in *Brassica*, the salt stressed *PORCx* plants would likely to transform Pchlide more efficiently than that of WT plants. Consequently, the accumulation of Pchlide in salt-stressed *PORCx* plants was substantially lower than that of salt-stressed WT plants (Fig. 83). The Chl catabolic product Pheophorbide a is a photodynamic compound and generates $^{1}O_{2}$ (Kharbash and Tripathy unpublished). Due to salt stress degradation of Chl is accelerated leading to increased accumulation of Chl catabolic product pheophorbide a. However, in response to salt stress, the degradation of Chl a is higher in WT plants as compared to that of *Brassica PORCx* plants. Although, expression of Pheophorbide a oxygenase (PAO) and its protein abundance increases in salt-stressed plants (Kharbash and Tripathy, unpublished), it cannot handle excess pheophorbide produced due to stress-induced Chl catabolism. Therefore, the accumulation of pheophorbide a in stressed plants was higher than in WT plants (Fig. 84). Not only Chl biosynthetic intermediate Pchlide but also the Chl catabolic product pheophorbid a could act as photosensitizers leading to excess production of $^{1}O_{2}$, membrane lipid peroxidation, injury to the plasma membrane leading to increase electrolyte leakage and ultimate cell death. As the concentration of both photosensitizers Pchlide and pheophorbide a were smaller in the transgenic they had reduced $^{1}O_{2}$ production and consequent decrease in MDA production, electrolyte leakage and cell death (Fig. 83-88). Increased generation of $^{1}O_{2}$ resulted in injury to the thylakoid membrane leading to decreased Fv/Fm ratio.

Fo, Fm and Fv/Fm of WT plants decreased in salt stress (Fig. 78). However, the transgenic plants always had higher Fv/Fm ratio in stress condition than that of WT. There was comparatively less reduction in Fo and Fm of transgenic plants under same growth conditions. This was due to efficient phototransformation of Pchlide to Chlide by abundant POR enzyme in transgenic plants resulting in reduced accumulation of reactive oxygen species (ROS) more specifically $^{1}O_{2}$. Increased $^{1}O_{2}$ production in WT plants would have
accelerated degradation of PSII reaction center D1 protein (Trebst et al., 2002; Grubes et al., 2001). Well developed and less distorted thylakoid in Brassica PORCx plants resulted in higher photosynthetic efficiency i.e., Electron transport rate (ETR), yield of photosystem II (ΦPSII), photochemical quenching in transgenic plants than that of WT in stress condition as well as in normal growth conditions (Fig. 79-81).

Decreased accumulation of Chl intermediate Pchlide led to reduced production of $^1$O$_2$ (Fig. 85) resulting in loss of cell damage in Brassica PORCx transgenic plants than that of WT. Measurement of MDA production, an indirect measurement of lipid peroxidation and electrolyte leakage, that reflects on membrane integrity were estimated to monitor cell injury. Brassica PORCx plants had lower MDA production (Fig. 86) and electrolyte leakage (Fig. 88) i.e., less cell damage. WT plants also had higher anthocyanin content than that of Brassica PORCx plants (Fig. 87). Anthocyanin is produced in response to stress. Due to impact of salt-stress WT plants accumulated anthocyanin while irrigated with 150 mM and 200 NaCl. However, due to reduced ROS production in transgenic plants the perception of stress stimulus was less and therefore PORCx plants accumulated reduced anthocyanin (Fig. 87).

Our above results demonstrate that in optimum growth condition in the absence of any stress Pchlide content, $^1$O$_2$ generation, MDA production and electrolyte leakage were lower in Brassica PORCx plants than that of WT resulting in higher photosynthesis and increased plant productivity. Therefore, in stress conditions the PORCx plants had better and more favourable photosynthetic machinery to tolerate stress. These measurements clearly demonstrate that Brassica PORCx plants had tolerance to salt stress.

To understand the impact of salt stress on plant productivity and seed yield plants were grown in soil in greenhouse conditions rather than inside the laboratory. The EC value of ~17 dSm$^{-1}$ was maintained in soil after periodic addition of 150 mM NaCl or half-strength Hoagland solution to the soil. This experiment also allow us to grow the plants in up to flowering, fruit setting and grain filling and ultimately the grain yield. Moreover, continuous salt treatment i.e., irrigation of vermiculite grown plants with NaCl along with half-strength Hoagland solution would have resulted in high adsorption of NaCl to vermiculite leading to increased salt concentration in the root zone. Therefore, in green house condition plants were grown in soil and treated with salt (EC~17 dSm$^{-1}$) to understand the impact of PORC overexpression for tolerance to salinity. Brassica PORCx plants had more foliar growth and fruiting. Soil-grown potted Brassica PORCx plants showed tolerance to salt stress (150 mM, EC ~17 dSm$^{-1}$). After 6 weeks of stress the flowering was delayed by one week in WT
plants. Under identical conditions the *Brassica PORCx* plants flowered. After 10 weeks of application of salt, both WT and transgenic had fruits although the fruiting was delayed in WT plants (Fig. 89)

*AtPORC* overexpression in *Brassica* resulted increase in photosynthesis and plant productivity. Net photosynthetic rate of transgenic plants was higher in control as well as in stress condition (Fig. 90). Increased efficiency of photosynthetic electron transport rate (ETR) in *Brassica PORCx* plants at was not accompanied by any increase in the relative quantum yield of CO$_2$ assimilation. Their quantum yield of CO$_2$ assimilation remained almost similar. The light-saturated net CO$_2$ assimilation rate was higher in *Brassica PORCx* plants in normal growth conditions that matches with that of ETR data. Increased rate of respiration in the transgenic plants enhanced the light compensatation point and contributed to augmented crop growth. Due to salt treatment the light saturated photosynthesis rate declined by 10% in WT whereas in *Brassica PORCx* plants it was not affected. The quantum yield of photosynthesis in WT and *Brassica PORCx* plants was not affected due to salt stress (Fig. 90).

Higher productivity and per plant grain yield (37%) in transgenic plants was due to increased rate of photosynthesis in normal growth conditions (Fig. 91). In salt stress condition *Brassica PORCx* plants due to their increased photosynthetic rate (22%) had higher grain yield/plant (115%) and more number of seeds per plant than that of WT (Fig. 91). It was due to their longer grain filling duration that resulted in higher 1000 seed weight.