称号及び氏名	博士(農学)　Al-Taweel　Khaled
学位授与の日付	平成１９年９月３０日
論　文　名	「A Bacterial Transgene for Catalase Protects Translation of D1 Protein during Exposure of Salt-Stressed Tobacco Leaves to Strong Light（バクテリア由来カタラーゼによる強光・塩ストレス下でのD1タンパク質の保護に関する研究)」
論文審査委員	主査　和田野　晃 副査　北村　進一 副査　杉本　憲治

論文要旨

Aerobic organisms such as plants and animals require oxygen for life. However, oxygen can also be harmful when reactive oxygen species (ROS) are formed. Chloroplasts are the major source of ROS in plants where the superoxide radical (O_2^-) is produced by photoreduction of O_2 at photosystem I (PSI) and II, and singlet O_2 from triplet excited state chlorophyll. Hydrogen peroxide (H_2O_2) can originate, in turn, from the spontaneous or enzyme-catalysed dismutation of O_2^- by SOD. By contrast, plants have an active system to detoxify the oxidative damage. The ROS Scavenging System consists of various redox substances as antioxidant metabolites (e.g. ascorbate (ASA), glutathione (GSH), and carotenoids) and enzymes such as catalase (Kat), superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione redactase (GR), and the enzymes of the AsA-GSH cycle including ascorbate peroxidase (APX). The system seems to be very important for the survival of plants under unfavorable conditions.

These transgenic approaches to increase plant tolerance to oxidative stress have focused on the quantitative modification of the antioxidant machinery e.g. overexpression of ROS scavenging enzymes. The *Escherichia coli* catalase encoded by the *katE* gene, HPII, which has a higher affinity for H_2O_2 than plant catalase and does not require ascorbate for its stability, it has been introduced into the chloroplast of the model plant *Nicotiana tabacum*. However, the efficiency of the *katE* to protect higher plants from the photo-oxidative stress needs to be confirmed in different plant species. In the present chapter, I tried to generalize the use of *E. coil* catalase in the other

economical crop. The *Agrobacterium tumefacience*-mediated method was used to produce transgenic tomato plants expressing *katE* in the chloroplasts.

The pBI101-katE binary vector harboring *katE* gene derived from *Escherichia coli* and driven by the promoter of the Rubisco small subunit gene of tomato, rbcS3C, was isolated and molecular characterized then transformed into competent DH5*α* cells using the heat chock method. The binary vector was transformed into competent LBA4404 *Agrobacterium tumefaciens* cells by two ways; the first one by the triparental mating method, and the second via the electroporation method. The pBI101-katE construct was introduced into a dwarf tomato (*Lycopersicon esculentum* L.) via *Agrobacterium tumefaciens*–mediated transformation. The construct was also introduced into a tobacco (*Nicotiana tabacum* L.) by biolistic bombardment. The infected parts of leaves were regenerated following plant propagation protocol that depends on Murashige and Skoog, (1962) MS medium to get putative transformed plants, and the latter were screened and confirmed by PCR.

Light stress and salt stress are important environmental factors that limit plant growth and productivity. Strong light impairs the activity of the photosynthetic apparatus, in particular that of photosystem II (PSII), via a process known as photodamage or photoinhibition. Kyle et al. (1984) suggested that the primary damaging effect of light might be the impairment of the quinone-binding protein, which is now known as the D1 protein, in the PSII complex. Impairment of D1 results in disruption of the light-dependent separation of charge between P680 and pheophytin a, and this phenomenon is associated with interruption of the transport of electrons that is mediated by PSII. However, photodamaged PSII can be repaired, and the repair process involves the rapid turnover of D1, with degradation of damaged D1 and subsequent light-dependent synthesis *de novo* of the precursor to D1 (pre-D1). The damaged D1 is replaced by newly synthesized pre-D1 from which a carboxy-terminal sequence is then removed by specific lumenal proteases. In the field, under natural conditions, salt stress very often occurs in combination with light stress, and several reports have appeared on the effects of salt stress on PSII under light stress. Salt stress apparently enhances the inhibition by strong light of PSII in *Chlamydomonas reinhardtii*, in leaves of barley, sorghum, rye, and in *Spirulina platensis*.

The synergistic effects of various stresses on photoinhibition have been studied extensively in cyanobacteria. However, cyanobacteria do not contain chloroplasts, and D1 of *Synechocystis* is encoded by a small family of *psbA* genes, namely, *psbA*2, *psbA*3 and *psbA*7. By contrast, in plants and eukaryotic algae, the *psbA* exists as a single-copy in the chloroplast genome. Moreover, the ROS-scavenging system of higher plants differs from that in cyanobacteria. Thus, results from cyanobacteria cannot necessarily

be applied to higher plants. In plants, the synergistic effects of light stress and other kinds of stress, such as salt stress and oxidative stress have not been characterized. In previous studies, we introduced the *katE* for catalase of *Escherichia coli* into tobacco and into tomato, with cDNA for a transit peptide that allowed the resultant catalase to enter the stroma of chloroplasts.

The primary aim of the present study was to investigate the effects of salt stress on photoinhibition in a higher plant and to elucidate the mechanism by which ROS in chloroplasts are involved in the stimulation of photoinhibition under salt stress. We also examined whether the conclusions derived from studies, in cyanobacteria, of the effects of salt stress on photoinhibition and the turnover of D1 can be applied to higher plants.

We examined the effects of salt stress (50 mM, 0.5 M or 1M NaCl), on PSII activity (Fv/Fm) during exposure of tobacco leaf discs from wild-type and *katE*-transformed plants to light stress (2,000 μE m^{-2} s^{-1}) for 3 h. Results demonstrated that salt stress enhanced the photoinhibition of PSII and that *katE*-transformed leaves were more tolerant to salt stress than were wild-type leaves. In darkness, incubation of leaf discs in the presence of 1.0 M NaCl drove minimal inactivation of PSII in either type of plant illustrating that, although exposure of leaf discs separately to light stress and to salt stress inactivated PSII minimally, combination of the two kinds of stress induced marked inactivation of PSII, revealing apparent synergism between the effects of strong light and high concentrations of NaCl.

To examine the contribution of *de novo* synthesis of chloroplast genome-encoded proteins to the salt stress-enhanced photoinhibition of PSII, we treated leaves with lincomycin. Results show that inhibition of protein synthesis by lincomycin dramatically accelerated the inactivation of PSII in leaf discs from both wild-type and transgenic plants. Moreover, the inactivation observed in the presence of lincomycin was also unaffected by the concentration of NaCl. However, the extent of inactivation in the presence of lincomycin was only minimal when leaf discs were incubated in darkness in the presence of 1.0 M NaCl in both types of plant. These observations suggested that the *de novo* protein synthesis might be involved in the synergistic effects of light stress and salt stress during the inactivation of PSII.

To examine the effects of NaCl on the level of D1 during photoinhibition, western blotting was preformed. The level of D1 decreased as the concentration of NaCl was increased from both wild-type and transformed plants. Aside from that, differences

between levels of D1 were insignificant in the both types at the same salt concentration.

We examined the effects of NaCl on *de novo* protein synthesis by monitoring the incorporation of [^{35}S]Met into proteins in thylakoid membranes in quantitative detail. We found that the translation of the *psbA* transcript to yield the D1 protein in leaves from wild-type plants was markedly suppressed in the presence of 0.5 M NaCl, while such translation was inhibited to a lesser extent in leaves from *katE*-transformed plants under the same conditions.

To identify the step(s) in the *de novo* synthesis of D1 that was inhibited by high concentrations of NaCl, we examined the effects of NaCl on the level of the transcript of the *psbA* gene during photoinhibition. The level of the *psbA* transcript increased rapidly at 50 mM NaCl. The final level of the transcripts at 0.5 M NaCl were almost unaffected in leaf discs from both wild-type and *katE*-transformed plants. At 1.0 M, NaCl almost completely prevented any increase in the level of the transcript. Thus, the *katE* transgene had no significant effect on the level of the *psbA* transcript under all tested conditions.

Our results indicate that inhibition of translation might be mainly responsible for the decrease in *de novo* synthesis of D1 at 0.5 M NaCl in wild-type tobacco plants. The catalase expressed from the transgene increased the efficiency of translation in katE-transformed plants, as compared to wild-type plants, by protecting the translational machinery in chloroplasts from attack by H_2O_2 under conditions of environmental stress. In addition, the findings of the present study are in almost full agreement with results obtained in cyanobacteria, suggesting that similar mechanisms might operate in plant chloroplasts and in cyanobacterial cells during the salt stress-induced inhibition of translation.

審査結果の要旨

光合成生物は水ストレス、強光ストレスなどを受けると、活性酸素種を生成し、光合成を行う種々の構成成分の酸化により、その能力を失うことが知られている。現在まで、活性酸素の初期ターゲットは、光化学系 II の構成成分である D1 タンパク質であると考えられてきた。

一方、基礎生物研究所の村田等は、らん藻で塩ストレスおよび強光ストレスにより発生する活性酸素の初期ターゲットには、D1 タンパク質だけではなく、その新規生合成系内のタンパク質も含まれることを明らかにしていた。本研究では、植物でも同様の機構がその光合成能力の低下に関与しているとの仮説を立てている。その証明には、クロロプラストのストロマに形質転換でカタラーゼが導入されている植物を用いることにより、膜タンパク質である D1 タンパク質とストロマに可溶化状態で存在している D1 タンパク質新規生合成系の活性酸素による攻撃を差別化し検討できると推測した。活性酸素の初期ターゲットが膜タンパク質である D1 タンパク質であるとすると、ストロマに可溶化状態で存在するカタラーゼの保護効果は低いが、ストロマに可溶化状態で存在している D1 タンパク質新規生合成系に対する保護効果は高いことが期待される。これらのことを踏まえて、申請者は、ストロマにカタラーゼを発現している形質転換植物の作出を検討し、トマトとタバコで目的の形質転換体を得た。

タバコ形質転換体を用い、塩ストレスおよび強光ストレス下で、光化学系IIの安定性、D1 タンパク質のチラコイド膜中での安定性、D1 タンパク質mRNAのレベル、D1 タンパク質の新規生合成速度を検討している。結果、0.5 M NaCl存在下、2,000 μEの強光をあてると、野生種のタバコでは急速な光化学系IIの活性低下が生じるが大腸菌カタラーゼ形質導入タバコではその低下が押さえられた。D1 タンパク質抗体でチラコイド膜中のD1 タンパク質量を検討すると、両者では活性低下の差を説明できるほどのD1 タンパク質量に違いは見られなかった。^{35}S-メチオニンの取り込みで検討したD1 タンパク質の新規生合成は野生種では非常に低いが、形質転換体では 50 mM NaCl存在下で見られた取り込みの 50%程度の値を観察している。またNothern BlotでD1 タンパク質mRNAを定量したところ野生種と形質転換体でそのレベルに差は見られず、0.5 M NaClストレス下でもむしろ増加の傾向が見られている。野生種と形質転換体で光化学系IIの活性低下に差が見られる条件下で、D1 タンパク質抗体によるチラコイド膜中のD1 タンパク質検出で差が見られないのは、機能しているタンパク質と機能を失ったタンパク質の差別化がWestern Blotでは不可能であり、mRNAレベルに差が無いにもかかわらず、野生種と形質転換体のD1 タンパク質新規生合成に差があること等を考慮し、次のように結論している。塩ストレスおよび強光ストレスによる植物の光合成傷害は、発生した活性酸素により、D1 タンパク質活性だけではなくその新規生合成能に傷害が生じた結果による。

以上、本研究は、高等植物光合成の塩ストレスおよび強光ストレス傷害に、D1 タンパク質とその新規生合成能の活性低下がともに関与していることを明らかにした。これらの成果は、植物生理学・生化学の発展に寄与するものであり、最終試験の結果と併せて博士（農学）の学位を授与することを適当と認める。