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学 位 論 文 題 目	Amelioration of oxidative stress generated by high salt,drought and sulfite using transgenic approach （植物遺伝子組換えによる高塩、乾燥、亜硫酸による酸化ストレスの緩和）
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学 位 論 文 の 内 容 の 要 旨

Almost all aspects of plant activities are affected by salt, drought stress and environmental pollutants such as sulfur dioxide and sulfur containing compounds, directly or indirectly. Although the nature of damage inflicted by high salt concentrations is not fully understood, but the integrity of cellular membrane, function of photosynthetic apparatus and the activity of various enzymes are affected by the toxic effect of high salts. As other biotic, abiotic and xenobiotic stresses, salt and drought stresses produce many degenerative reactions mediated by toxic reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen and hydroxyl radical　which is produced when the H_2O_2 react with reduced metal irons, such as Fe, (Halliwell and Gutteridge 1989). These ROS are cytotoxic and can seriously disrupt normal metabolism through oxidative damage to lipids (Fridovich 1986 , Wiseand Naylor 1987, McKersie and Leshem　1994]), nucleic acids (Fridovich 1986 , McKersie and Leshem 1994, Imlay and Linn 1988) and protein (McKersie and Leshem 1994, Davis 1987), resulting in mutation, protein destruction and peroxidation of membrane lipids, respectively, which in turn may result in diseases and degenerative processes (carcinogenesis, immunodeficiency and aging), Chloroplasts are especially prone to generate ROS because O_2 concentration is high during photosynthesis (Steiger et al 1977) and the overreduction of photosynthesis become inevitable . Plants use diverse array of antioxidant enzymes such superoxide dismutase, ascorbate peroxidase, catalase, glutathione peroxidase, glutathione S-transferase and other low molecular weight antioxidant such as reduced glutathione and ascorbic acid, to alleviate the oxidative damage by scavenging the ROS.

Progress has been made in identification and characterization of the mechanisms that render plants tolerant to high salt. Of these mechanisms, is understanding of metabolic pathways and the problem encountering the production of compatible solutes. Salinity is severely affecting the plants by imposing water deficit due to the high ions concentration, and also through ion-specific stress. Salt-sensitive plants (glycophytes) and salt-tolerant plants (Halophytes) have similar sensitivity to salt in their cytosolic enzymes, so the maintenance of high K^+/Na^+ ratio in the cytosol is key role for improved performance under high salt concentration. Thus, engineering the accumulation of salt in vacuoles and the extrusion of Na^+ from young leaves and meristematic tissues will maintain high K^+/Na^+ ratio. Synthesis and accumulation of osmoprotective substance known as compatible solutes, is one of the cellular responses of organisms to salinity. These compounds can increase cellular osmotic pressure by utilizing protein and other cellular structures, and therefore, providing protection from short term water stress. The most recent examples is the engineering of ectoine synthesis in plants (Ono et al 1999), trehalose synthesis in plant (Goodijin and Van Dun 1999) and manipulation of sucrose metabolism by expressing yeast invertase in tobacco plants (Fukushima et al 2001). But proline and glycine betaine received more attention (Rontein et al 2002), although the production of free proline is not straightforwards since proline is catabolism by proline dehydrogenase, which is upregulated by free proline, is common (Roosens et al 1999). Improvement of tolerance to salinity was correlated to the level of free proline in the cells. Glycine betaine is receiving more attention than any other compatible solute as it is not significantly degraded in plants like proline (Nuccio et al 2000). Glycine betaine is synthesized in chloroplasts from choline in two oxidation reduction reactions, first oxidation to betaine aldehyde which catalyzed by choline monooxygenase and the second step betaine aldehyde oxidation to glycine betaine catalyzed by betaine aldehyde dehydrogenase, but in some organisms choline oxidase can carr the production of glycine betaine in one step. This allowed the engineering of glycine betaine in Arabidopsis thaliana with improvement in tolerance to salinity, drought and freezing. Glycine betaine can accumulate to high levels (more than 20mM) in some plant species such as sugar beet and spinach under osmotic stress.

Chloroplast, which is the compartment of a high energetic reactions of photosynthesis and a generous supply of oxygen, is a rich source of reactive oxygen species (ROS), such as superoxide radical , hydrogen peroxide, singlet oxygen and hydroxyl radical (Asada 1997). Hydrogen peroxide concentration, as low as 10 μM, can inhibit photosynthesis by up to 50% (Kaiser 1979), because it inactivates thiol-regulated enzymes (Tanaka 1994). Moreover, H_2O_2 can react with reduced metal ions, such as Fe, producing the hydroxyl radical, which is the most harmful, attacking membrane lipids, causing base mutations, breakage of DNA strands and alteration of proteins (Bowler et al 1992). The formation of ROS in the chloroplasts is enhanced when carbon assimilation is inhibited (Camp et al 1996). For review see Halliwell (1987), Cadenas (1989) and Fridovich (1989). Efficient removal of ROS from the chloroplast is a key factor for normal metabolism in plants. The major ROS scavenging enzymes in plants include superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (Asada 1999). Detoxification of ROS in stressed plant is proved to improve the capability of plants to withstand the adverse stress conditions, especially when these protectants were targeted to the chloroplast, more details will be found in inside pages. Prevention or alleviation of damage is one of the strategies used to achieve salt tolerance, the second one is re-establishment of new homeostatic condition in the affected plants, and the rate of growth must be controlled.

Due to human activities the concentration of SO_2 in the atmosphere has greatly increased. The reduced form of sulfur plays an important role in biosynthesis of primary and secondary metabolites, but both dry and wet deposition of SO_2 caused severe damage to plants and other ecosystem. During peak concentration of SO_2, it will reach toxic levels to which the plants will respond by activation of various defense mechanisms. One such response is antioxidant defense system. Stomatal closure in plant to avoid absorption of SO_2 is also one of such mechanisms, but it accompanied with decrease in photosynthesis by limiting CO_2 concentration. In the early investigations the higher stomatal resistance was correlated to the tolerance to acute SO_2 injury, which dissolved in cellular solution forming sulfite bisulfite. Direct metabolism of the product of SO_2 in the plant could be the rapid metabolism of sulfite, which could either be through the oxidation of sulfite to sulfate or its reduction to sulfide. Plants with higher sulfite oxidation showed high degree of tolerance to SO_2 (Ayazloo et al 1982). Ferredoxin NADP reductase was found to oxidized sulfite but with the concomitant production of oxygen radicals (Nakamura 1970), so evading sulfite toxicity and trapping in oxygen radicals will not be considered as detoxification. In animal hepatic sulfite oxidase can do the job without production of oxygen radicals (Cohen et al 1973), but this enzyme is not fully characterized in plants. Genetic manipulations of plant for SO_2 tolerance will be essential for food production and bioremediation for the environment.

The role of SOD and APX in protecting the plants will be reported in the first part of this work, in the second part the sulfite oxidase transgenic plants will be evaluated for its role in protecting plants from SO_2 toxicity.

論 文 審 査 の 結 果 の 要 旨

本論文は乾燥､高塩耐性植物を作出するために､活性酸素消去系の鍵になる２酵素の遺伝子組換えを行い、耐性を調べた研究である。以下の研究の概要を示す。

乾燥、高塩耐性植物を遺伝子組換えで作出する上で、活性酸素消去に関わる酵素は有力な遺伝子である。植物が乾燥、高塩ストレス下に置かれると、気孔が閉鎖し、二酸化炭素吸収が抑制され、光化学系に過剰還元力が蓄積され、活性酸素濃度が増加する。乾燥、高塩ストレス時、植物は、初期には光合成の低下など生理レベルの変化、その後、萎れ、葉の変色、枯死などの可視障害を受ける。活性酸素は葉の変色、枯死など不可逆的な影響だけではなく、ストレス初期の代謝阻害、萎れなどにも関与していると思われる。活性酸素消去系を強化すれば､乾燥･高塩時の初期障害を防ぐだけではなく､萎れから、枯死に至る期間を延ばす、つまり、水供給が少々遅れても回復できる植物の作出が可能となる。

植物は巧妙な活性酸素消去系を進化的に獲得した。活性酸素（O_2^-スーパーオキシドラジカル、H_2O_2：過酸化水素、$^1O_2$：１重項酸素、OH・：ヒドロキシルラジカルなどの総称）の主な生成機構は

光合成光化学系やミトコンドリアの電子伝達系の構成成分の中の高い還元力を持つ物質から、酸素分子への電子流出である。酸素が1電子還元されると、O_2^-が生成する。O_2^-は活性酸素消去系において中心的な役割を果たすスーパーオキシドジスムターゼ（SOD）により、H_2O_2に変化する。H_2O_2はペルオキシダーゼ（植物の場合はアスコルビン酸ペルオキシダーゼ（APX）により水に無毒化される。もし、O_2^-やH_2O_2がSODやAPXの許容量よりも多く生成蓄積すると、これらの活性酸素よりもはるかに強毒性の$^1O_2$やOH・を産出し、,細胞に致死的な障害をもたらす。植物は多くの活性酸素消去物質を含有するが、SODとAPXは特に重要な役割を担っていると思われる。本研究では、活性酸素消去系の根幹をなす2つの酵素を活性酸素生成量の多い葉緑体で高発現させることにより、乾燥・高塩ストレスに耐性を持つ植物を作出することを試み、その性質について検討した。

イネの細胞質型Cu、Zn-SODcDNA及びアラビドプシスの細胞質型APXcDNAをアラビドプシス葉緑体グルタチオン還元酵素cDNAの葉緑体トランジットペプチド遺伝子の下流に連結して、植物高発現型プラスミドベクターpBE2113にサブクローニングし、アグロバクテリウムを介して、タバコに遺伝子を組込んだ。このタバコのゲノムDNAへの組込み、葉緑体での発現、各種環境トレス（乾燥・高塩、パラコートと亜硫酸など）応答などを調べた。

SOD遺伝子、APX遺伝子のタバコゲノムDNAへの取込みがサザンドットブロッティングで確認された。SOD遺伝子組換えタバコにおいてSOD酵素活性が非組換えタバコよりも高いこと、電気泳動により分離後活性染色することにより、イネのSODがタバコで発現していることを確認した。組換えタバコ葉から葉緑体を単離することにより、SODが葉緑体で発現していることを確認した。APX組換えタバコについても同様にアラビドプシスAPXが葉緑体で発現していることが確認できた。

興味あることに、SOD組換えタバコは予想通りSOD活性が高かったが、APX活性が高かった。また、APX組換えタバコは予想通りAPX活性が高かったが、SOD活性も高かった。活性酸素毒性を効率良く防御するにはO_2^-とH_2O_2を同時に迅速に消去する必要がある。植物は両酵素を同時に高発現させる機構を備えているのかも知れない。

SOD形質転換タバコ、APX形質転換タバコの各種ストレス耐性について検討した。両植物を0.3M食塩処理した時の影響を光合成により調べた時に、非組換え植物よりも耐性を示すことを確認した。途中で食塩処理を停止した時の回復も両組換え植物が高いことを見い出した。生長速度も明らかに、組換え植物が高いことが判明した。水供給を停止することによる乾燥ストレス、ポリエチレングリコール処理の影響も調べたが、同様に両組換えタバコは耐性を示した。

これらの結果から、SOD形質転換、APX形質転換両タバコとも、乾燥・高塩ストレスにより引き起こされる光酸化ストレス耐性を獲得レたことが証明された。今後は活性酸素消去以外のメカニズムによって乾燥・高塩ストレスに関わる遺伝子を組換えた植物（例えば適合溶質蓄積植物、ナトリウムポンプ強化植物など）などと交配等で複合で遺伝子発現させることにより、より乾燥地での生育に適した耐性植物の開発が可能になると思われる。

よって、本研究論文は博士（農学）の学位論文として十分な価値を有するものと判断した。