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INCREASED RESISTANCE OF *ARABIDOPSIS CPR5* MUTANT TO H_2O_2- INDUCED PHOTOOXIDATION

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Abstract

*cpr*5 is predicted to be a putative transmembrane protein involved in several cellular processes, including signal transduction plant defense and trichome development. The mutation of the *cpr5* gene increases the resistance of *Arabidopsis* to pathogens. In this study, we show an *In vitro* increased resistance of *cpr*5 leaves to hydrogen peroxide-induced photooxidation. Both fluorescence parameters (including Fv/Fm, $\phi_{PS\square}$, qP and NPQ) and the activities of two anti-oxidative enzymes (SOD and APX) were used to evaluate the response of mutant and wild-type leaves to H_2O_2 treatment. During a 360-min., treatment, both mutant and wild-type leaves showed a time course dependent decrease trend in Fv/Fm values. However, the decrease rate for mutant leaves (0.046/min) was nearly twenty five-fold lower than that for wild-type leaves (0.101/min). The leaves of *cpr5* dramatically delayed the reduction of $\phi_{PS\square}$ values and showed a different profile of $\phi_{PS\square}$ from the wild-type. At 240 min., of treatment, the value of $\phi_{PS\square}$ for the mutant leaves was nearly six-fold as that of the wild-type. The rates of cellular membrane leakage were constitutively lower in the *cpr5* leaves than in the wild-type. In addition, the *cpr5* leaves showed slightly higher activities of the SOD and APX enzymes than did the wild type. These results indicated that *cpr5* mutant increased both anti-oxidative capability and the stability of PSII to H_2O_2-induced photooxidation.

Abbreviations: APX, ascorbate peroxidase; AsA, ascorbic acid; CAT, catalase; Fv/Fm, maximal PSII quantum yield; GSH, glutathione; NPQ, non-photochemical quenching; qP, coefficient of photochemical quenching; SA, Salicylic acid; SAR: systemic acquired resistance; SOD, Superoxide dismutase; PVP, Poly Vinyl Pyrrolidone; ϕ_{PSII}, effective PSII quantum yield.

Introduction

The function of the *cpr*5 gene is hypothesized to involve in several processes including signal transduction, cell proliferation, cell death and pathogen-defense responses. It was reported that the expression level of the *cpr*5 gene was increased in the beginning of pathogen infection to *Arabidopsis* (Yoshida *et al*., 2002). Recently, the *cpr*5 gene was proposed to encode a novel putative transmembrane protein, which contains five (pathogenesis-related genes) putative transmembrane helices at the C terminus. In addition, *cpr*5 is predicted to be a Type IIIa membrane protein, due to a PSORT structure identified at the N-terminus (Kirik *et al*., 2001). It was observed that mutations in *cpr*5 show pleiotropic effects on the regulations of cell death, cell elongation and trichome development (Kirik *et al*., 2001; Yoshida *et al*., 2002).

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The *cpr5* mutant was identified from a screen for constitutive expression of systemic acquired resistance (SAR) (Bowling, *et al*., 1997). SAR is a plant defense response of an acquired systemic resistance to subsequent pathogen infection, after a plant is infected by an avirulent or other necrotizing pathogen (Ross, 1961; Kuc, 1982). The *cpr5* mutant was characterized by a spontaneous pathogen-defense response and a constitutive express of SAR**.** These phenotypes were explained to result from the accumulation of reactive oxygen species, the induced expression of PR-1 genes (pathogenesis-related gene 1) and elevated levels of SA (Boch, *et al*., 1998). The influence of salicylic acid (SA) on plant growth, short-term acclimation to high light (HL) and on the redox homeostasis of *Arabidopsis thaliana* leaves have been reported recently (Mateo, *et al*., 2006). However, little is known about the response of the *cpr5* mutant to photooxidation. Hydrogen peroxide (H_2O_2) is a highly reactive oxygen species. It has strong oxidizing properties (Salin, 1988). In this study, we found an increased resistance of the mutant plants to H_2O_2-induced photooxidation.

Materials and Methods

Plant materials and growth conditions: Seeds of *Arabidopsis thaliana*, ecotype Columbia (Col) and *cpr*5 mutant were obtained from the *Arabidopsis* Biological Resource Center in America. Seeds were imbibed for 2 d at 4^{o}C in the dark to synchronize germination, and then sown on sterilized compost. Plant seedlings were grown routinely in a growth cabinet at 20-22^{o}C with a 16-h photoperiod (100 μmol m^{-2} s^{-1}) and a relative humidity of 80%. The third and the fourth mature leaves from 28 d-old plants were used for experiments.

Photooxidative treatment of leaves: The detached leaf discs (diameter: 5mm) cut from the *cpr*5 mutant and wild type plants were soaked in a solution of 100 mmol/L H_2O_2 (with 0.01% (v/v) TritonX-100) after vacuum infiltration for 10 min. Leaves in distilled water were taken as the control. The treatments were conducted in the growth chamber (RXZ-500, Ningbo Jiangnan Instrument Factory, China) with the temperature of 25^{o}C and a light intensity of 90 μ mol m^{-2} s^{-1}.

Cell membrane leakage rate: Leaves (0.2 g) of two phenotypes were immersed in double distilled water for 1.5 h at room temperature, followed by a 30 - min boiling treatment, respectively. The conductibility of leaky electrolyte before and after boiling was determined using a DDS-11 A conductometer (Shanghai Dapu Instruments Limited Company, Shanghai, China).

The determination of antioxidase activity: The crude enzyme solution was prepared as follows: 0.2 g leaves were ground in ice bath with 50 mmol/L phosphate buffer (pH 7.0, with 1% PVP), followed by centrifugation at 15 000g and 4^{o}C for 20 min., the supernatant was collected for enzyme assay. Superoxide dismutase (SOD) activity was determined based on the inhibition of reduction of nitro-blue tetrazolium in the presence of riboflavin in the light at 560 nm as described by Giannopolitis & Ries (1977). A unit of SOD activity is defined as the amount of enzyme, which caused 50% inhibition of the reaction in the absence of enzyme. The activity of ascorbate peroxidase (APX) was measured according to Shen *et al*., (1996). The assay was performed in a reaction mixture containing 50 mmol/L phosphate buffer (pH 7.0), 0.5 mmol/L AsA, 0.1 mmol/L H_2O_2 and 0.1 ml enzyme extract. The change in A_{290} was recorded for 1 min., after the addition of ASA using an enzyme kinetics software in a spectrophotometer (Lambda 25, Perkin-Elmer Inst, USA).

Chlorophyll fluorescence measurements: Fluorescence measurements were carried out with an IMAGING-PAM chlorophyll fluorometer (Heinz Walz GmbH, Effeltrich, Germany) as described by Siebke & Weis (1995) and Rascher *et al*., (2001). Fluorescence was measured with relatively weak measuring light pulses (<1μmol m^{-2} s^{-1}) at a low frequency (1 Hz) for measurement of Fo. Fm was measured during a 800-ms exposure to a PPFD of approximate 2,700μmol m^{-2} s^{-1}. The intensity of continuous actinic illumination was adjusted to 185μmol m^{-2} s^{-1}. All lighting (modulated measuring light, actinic light and saturation pulses for measurement of Fm and Fm') is provided from blue light-emitting diodes (450 nm). All fluorescence measurements were started after an additional 10-min., dark adaptation. When performing a measurement, an area of interest (AOI, diameter: 1cm) was selected in the middle of the whole leaf. Values of the chlorophyll fluorescence parameters Fo (minimum fluorescence yield of dark-adapted leaf), Fm and Fm' (maximal fluorescence yield of dark-adapted and light-adapted leaf), Fv/Fm (maximal PS II quantum yield), Φ_{PSII} (effective PS II quantum yield), NPQ (nonphotochemical quenching) and qP (coefficient of photochemical quenching) were the average of the AOI. In addition, their images were simultaneously derived from the IMAGING-PAM software. The resulting values of these fluorescence parameters were the averaged values of the circulated areas. Non-photochemical quenching (NPQ) was normalized by dividing by 4, keeping values between 0-1.The images of these fluorescence parameters can reflect heterogeneity of photosynthetic activity of the whole leaves. At the bottom of the image window, the false color code bar is located. The standard false color code ranges from black via red, orange, yellow, green, blue and violet to purple. These colors code for numbers is between 0 and 1. Hence, all measured or calculated parameters are normalized to values between 0 and 1.

Statistical analysis: All of the data shown were from three or five measurements. Statistical analysis was conducted by Origin 7.5 software (Microcal Company, Northampton, USA). Values after ± show standard errors.

Results and Discussion

Changes of Fv/Fm in leaves of the mutant and wild type exposed to H_2O_2-induced photooxidation: Chlorophyll fluorescence parameters for Fv/Fm were measured and their images were recorded simultaneously at 13 time point during the 360 min treatment. The values of Fv/Fm in leaves of both the *cpr5* mutant and Columbia ecotype constitutively deceased (Fig. 1A). The decrease rate of Fv/Fm in leaves of WT was estimated to be 0.101/min. However, the decrease rate for *cpr*5 mutant was approximate by 0.046/min. In addition, the recorded images of Fv/Fm for leaves of WT and *cpr*5 mutant strongly supported this result (Fig. 1C). During 360-min., treatment of photooxidation, images of Fv/Fm in WT faded gradually from blue (0.8) to red with partial orange (0.1~0.2), indicating that the quantum efficiency of light energy transfer in PSII was close to 0.1 (Fig. 1C). In contrast, images of Fv/Fm for leaves of *cpr*5 mutant showed relatively long time of blue (0-60 min.,), blue-green (90-24 min.,) and green (360 min.,) colors, suggesting that the PSII in leaves of *cpr*5 mutant be more stable. Clearly, the relatively rapid decrease of photochemical efficiency of PSII in WT revealed that *cpr*5 mutant had higher tolerance to oxidative stress than the WT plant.

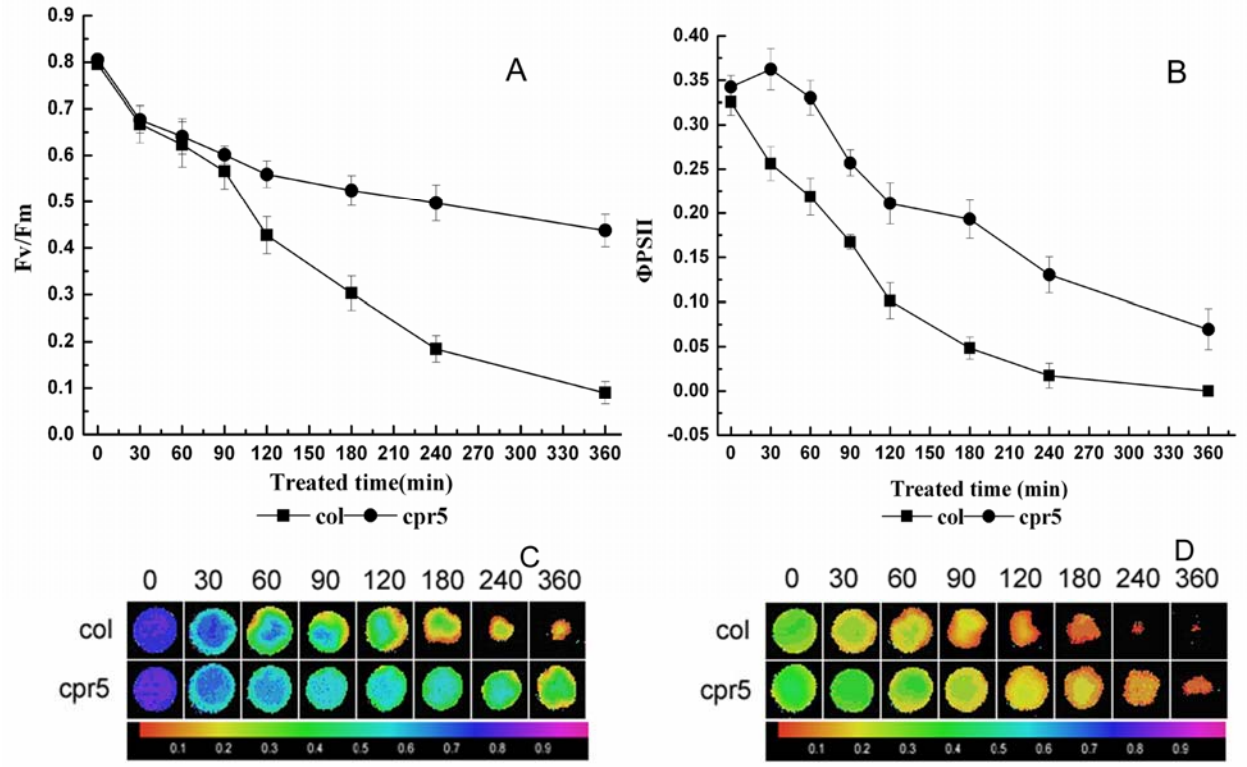

Fig. 1. Responses of *cpr5* and wild-type *Arabidopsis thaliana* to photooxidation induced by hydrogen peroxide (H_2O_2) *In vitro* are shown by values of Fv/Fm (A) and ΦPSII (B), as well as by fluorescence images from both Fv/Fm (C) and Φ PSII (D) recorded from leaves. Fluorescence images was indicated by color code in the order of black (0) through red, orange, yellow, green, blue, violet, to purple (1). The number codes underlying images are marked from 0 to 1, showing the changes.

Changes of ϕPSII Values in leaves of the mutant and wild type exposed to H_2O_2-induced photooxidation: ϕPSII is the other parameter used to show the actual efficiency of light energy capture under the close of partial reaction centers in PSII (Krall & Edward, 1992). The changing tendency of ϕPSII values was different in leaves between the mutant and wild-type *Arabidopsis* in responding to photooxidation induced by H_2O_2. The values of ϕPSII for leaves of WT rapidly declined from 0.33 to 0.025 within the 240 min., but similar between the two time points of 240 and 360 min., (Fig. 1B). In contrast, the ϕPSII value for leaves of the *cpr*5 mutant at 30 min., was slightly increased. Then, a decrease trend of the ϕPSII values was observed from 30 min., to 360 min., (Fig. 1B). In comparison with the wild-type leaves, the decrease rate of ϕPSII values for the *cpr*5 mutant was smaller. This observation was supported by recorded images of chlorophyll fluorescence. The *cpr*5 mutant delayed the fading time of green color. It was clearly observed that the green fluorescence was replaced by red at 180 min., in the wild-type leaves, but the time was postponed to 360 min., for the mutant (Fig. 1D). These results provided data to show an increased resistance of the mutant to photooxidation. The rapid decease in ϕPSII indicated that PSII was damaged severely by oxidative stress. Changes in these two chlorophyll fluorescence parameters (Fv/Fm and ϕPSII) and their images (Fig. 1C, D) revealed that WT was damaged more severely than *cpr*5 mutant.

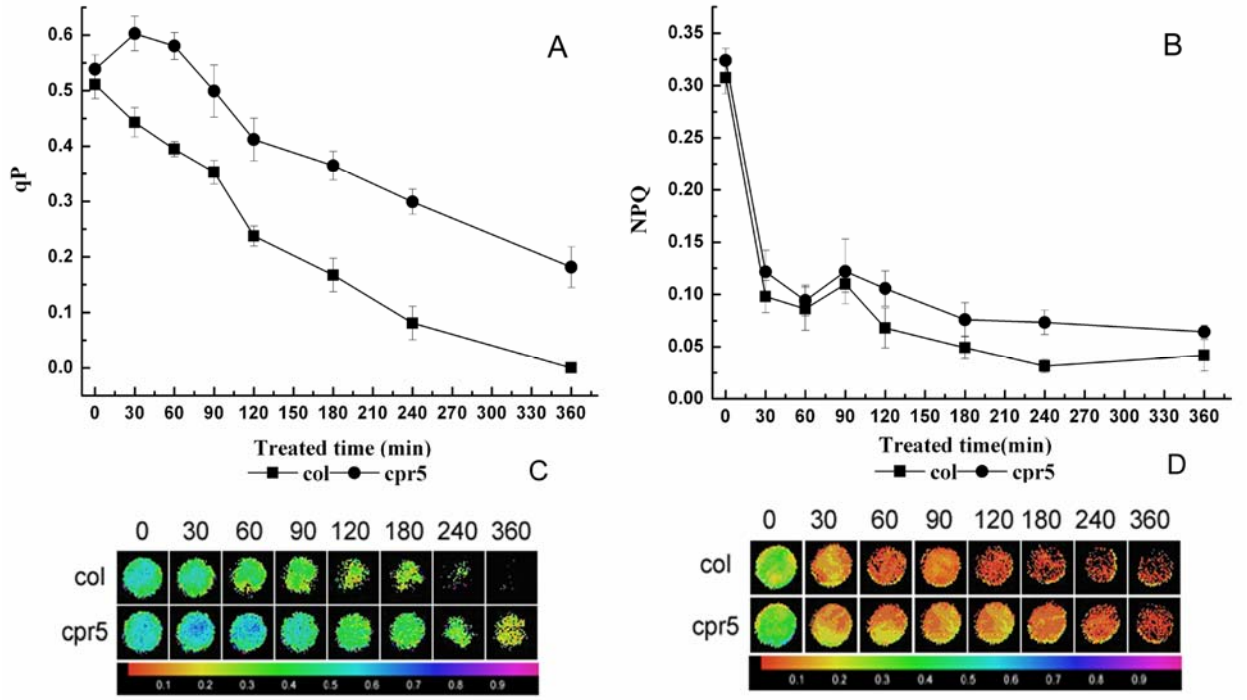

Fig. 2. Responses of *cpr5* and wild-type *Arabidopsis thaliana* to photooxidation induced by hydrogen peroxide (H_2O_2) *In vitro* are showed by values of qP (A) and NPQ (B), as well as by fluorescence images from both qP (C) and NPQ (D) recorded from leaves. Fluorescence images was indicated by color code in the order of black (0) through red, orange, yellow, green, blue, violet, to purple (1). The number codes underlying images are marked from 0 to 1, showing the changes.

Changes of qP and NPQ values: qP (coefficient of photochemical quenching) is used to indicate the proportion of open reaction centers in PSII (Genty *et al*., 1989). The values of qP for the wild-type leaves were rapidly decreased from approximate by 0.5 at 0 min., to nearly zero at 360 min (Fig. 2A). In contrast, the qP value was slightly higher at 30 min., than at 0 min., for the *cpr5* mutant and the delayed reduction of qP values were obviously observed. The qP value for the leaves of the *cpr*5 mutant at 360 min., was approximate by 0.2, similar to the value for the wild-type leave at 120 min., (Fig. 2A). Obvious different responses to H_2O_2-treatment were observed between the Columbia ecotype and the mutant (Fig. 2C). As shown in Fig. 2A, qP in leaves of WT decreased continuously under photooxidation, indicating that the proportions of open reaction centers in PSII and electrons involved in CO_2 fixation were decreased. However, qP in leaves of *cpr*5 mutant was increased slightly in the first 30-min., treatment, then decreased persistently. During 360 min., of photooxidative treatment, the capacities of photochemical quenching of PSII in leaves of *Arabidopsis* exhibited the sequence *cpr*5>WT ($p<0.01$). Change of imaging color was consistent with the numerical change of qP (Fig. 2C). NPQ is the abbreviation of non-photochemical quenching, which is used to reflect the dissipation capability of heat energy in plants (Hartel & Lokstein, 1995). Both the mutant and wild type plants showed similar trend for the NPQ profiles. During 360 min., of photooxidative treatment, changes for the values of NPQ were as follows: a rapid decrease (0~60 min.,), slight increase (60~90 min.,) and then slow reduction (90~360 min.,) (Fig. 2B). The recorded images of fluorescence supported this result (Fig. 2D). In comparison, the decrease rate of NPQ values from 90 min., to 240 min., was slightly smaller in the mutant leaves than in the wild-type leaves (Fig. 2B). The drastic decrease in NPQ indicated the loss of capability to dissipate heat energy and the photo-

protective potentials of both phenotypes induced by H_2O_2 in the light. As outline in qP and NPQ, a conclusion was obtained that the sensitivity of PSII to photooxidation was present by WT >*cpr*5 (qP: $p<0.01$; NPQ: $p<0.05$).

Membrane permeability and activities of SOD and APX: Membrane permeability is a relevant index that reflects the degree of impaired membrane function. At 0 min., the percentage of membrane permeability between the two group plants was similar (Fig. 3A). At 180 min., the membrane permeability was slightly lower in the mutant leaves than in the wild-type. At 360 min., the percentage of the membrane permeability in the wild-type was nearly 2-fold as that in the mutant leaves. This result showed that the mutation of the *cpr* gene increased the resistance of cellular membrane to H_2O_2-induced damage.

Then activities of two antioxidative enzymes, SOD (Giannopolitis & Ries, 1977) and APX (Shen *et al*., 1996) were estimated in leaves of the two group plants. Before photooxidation treatment (0 min.,), the two enzymes from the mutant showed slightly higher activities than that from wild-type control. In comparison with activities measured at 0 min., activities of the two enzymes at 180 min., and 360 min., were reduced by H_2O_2-induced stress. However, the activities of SOD and APX decreased more quickly in WT than in *cpr*5 mutant (Fig. 3B and 3C). The results suggested that the *cpr*5 mutant increased the antioxidative ability, thus enhanced the stability of plasma membrane.

Production of excessive reactive oxygen species (ROS) causes severe oxidative stress in chloroplasts and leaf cells, finally induces photooxidative injury. In the present study, photooxidation induced by exogenous H_2O_2 resulted in the increase of electrolytes leakage rate and the decrease of chlorophyll fluorescence parameter (Fv/Fm, ϕ_{PSII}, qP, and NPQ) in leaves of *cpr*5 mutant and WT, which suggested that photosynthetic apparatus had been obviously damaged.

Salicylic acid (SA) was accumulated in *cpr*5 mutant (John *et al*., 1995; Morris *et al*., 2000; Ryals *et al*., 1996). The activities of antioxdative enzymes could be enhanced by SA treatment. Rao *et al*., (1997) reported that SA treatments increased activity of Cu, Zn-SOD, but inactivated catalase (CAT) and APX. The activities of both SOD and APX increased, whereas CAT activity decreased under SA treatment in grape seedling (Wang *et al*., 2003). Our results showed that the activity of APX significantly increased in *cpr*5, and there is no difference in activity of SOD between WT and *cpr*5 (Fig. 3B and 3C). We proposed that the accumulation of SA possibly induced the increment of APX activity in *cpr*5. APX is the main enzyme located at chloroplast to degrade H_2O_2. This is a possible reason that *cpr*5 exhibited higher tolerance to photooxidation induced by H_2O_2 than WT.

On the other hand, SA treatment could induce the resistance of plants to environmental stresses by increasing the content of antioxidative substance such as GSH (Mateo *et al*., 2006), AsA (Shi *et al*., 2004) and polyphenolic compound (Cevahir *et al*., 2005). So we presumed that the content of antioxidative substance possibly increased in *cpr*5 mutant due to its accumulation of SA. These antioxidative substances can eliminate ROS to protect PSII against photooxidation induced by H_2O_2. This may be another reason for *cpr*5 mutant in possession of higher tolerance to photooxidation.

It was very clear that in comparison with WT, PSII in leaves of *cpr*5 mutant was more stable under photooxidative stress induced by exogenous H_2O_2, and *cpr*5 mutant exhibited higher antioxidative capability. However, further investigations are still required to understand its specific mechanisms of action and principles.

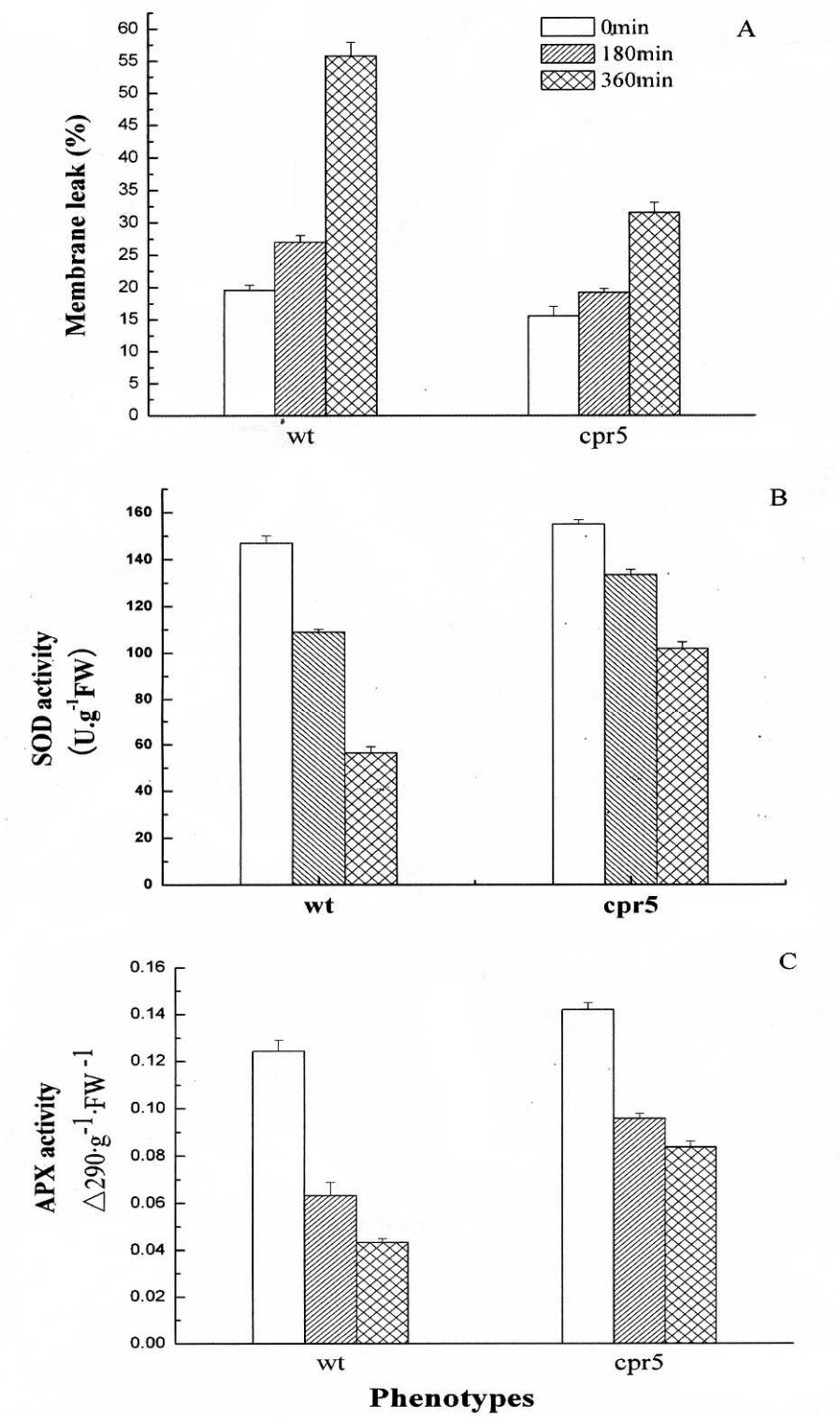

Fig. 3. Time course of cell cell membrane leakage rate(A), the activities of SOD (B) and APX (C) in leaves to photooxidation induced by hydrogen peroxide (H_2O_2).

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