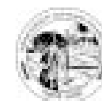
Indian J. Plant Physiol., Vol. 14, No. 3, (N.S.) pp. 209-214 (July-Sept., 2009)



RELATIONSHIP BETWEEN ANTIOXIDANT ENZYMES AND SELF- AND CROSS-POLLINATION IN THE STYLES OF TWO *PETUNIA HYBRIDA* CULTIVARS

OLOUMI HAKIMEH*[1] AND REZANEJAD FARKHONDEH[2]

[1]International Centre for Science, High-Technology and Environmental Science, Mahan, Kerman, Iran, [2]Biology Department, Science Faculty, Shahid Bahounar University, Kerman, Iran

Received on 26 Nov., 2008, Revised on 26 Sept., 2009

SUMMARY

The possible relation between self-incompatibility (SI) and the stress response using pistils after self- pollination was examined in two cultivars of *Petunia hybrida*. Hydrogen peroxide content, enzyme activities of stress enzymes were compared between self- and cross-pollinated styles of Bravo cool water mix (self-compatible) and Bravo purple star cultivar (self-incompatible) of *Petunia hybrida*. H_2O_2 content was significantly higher in self-pollinated styles of both cultivars. The activity of peroxidase (POD) and superoxide dismutase (SOD) was promoted by self-pollination in the styles of Bravo purple star cultivar. The activity of catalase (CAT) and ascorbate peroxidase (APX) were lower in self-pollinated styles in Bravo purple star cultivar. There were no differences between those pollinated styles of Bravo cool water mix cultivar and only the activity of SOD were higher in self-pollinated in this cultivar. Based on our results, it seems that there is a correlation between incompatible pollination and some stress enzymes activity in the style of *Petunia hybrida*.

Key words: **Enzyme activity, isoenzyme pattern, *Petunia hybrida*, self-incompatibility, stress enzymes.**

INTRODUCTION

Many plants possess determined SI systems through which genetically related-pollen is recognized and rejected prior to fertilization, leading to obligate out-breeding (Roalson and McCubbin 2003). *Petunia* has a strong gametophytic self-incompatibility (GSI) system (Shivanna and Sharma 1985) as *Nicotian*a (Bredemeijer 1976) and *Pyrus* (Tomimoto *et al*. 1996). GSI system in *Petunia* is controlled by a single, multiallelic S-locus (Shivanna and Sharma 1985). In GSI system, incompatibility reaction is determined by the haploid S-genotype of the pollen grain (Thompson and Kirch 1992). Growth of haploid pollen tube carrying an S-haplotype identical to one of the two S-haplotypes carried by the diploid pistil is inhibited in the style (Wang *et al*. 2003, Rudd and Franklin-Tong 2003, McClure 2006). Although in most cases SI is controlled by S-locus, recent results show that surprisingly complex signal transduction pathways and many players are involved in pollen recognition and rejection (McCormic 1998).

Rudd and Franklin-Tong (2003) stated that the postulated interaction of the pollen S-receptor triggers a signaling cascade(s) in incompatible pollen tubes that results in inhibition of tip growth. It has been reported that after self-incompatible pollination, a pistil can be considered to be subject to some kinds of stress (Tezuka *et al.* 1997, Suzuki *et al*. 2001, Tsuruhara and Tezuka 2001, Neog *et al*. 2004). Rejection of pollen tube growth is related to the synthesis of special enzymes that breakdown the incompatibility barriers. When organisms are subjected to stress, production of active oxygen

*Corresponding author, E-mail: oloomi2001@yahoo.com

species such as $O_2^{\bullet -}$, $^1O_2$, $OH^{\bullet}$ and H_2O_2 are enhanced in their cells. So regard SI as a physiological stress, it might be expected to induce high levels of free radicals in the cells of the stressed pistil. Growth inhibition of pollen tubes after self-incompatible pollination is related by high levels of active oxygen species that induce secondary stress and result from self-incompatible pollination. Self-incompatible pollination affects the activity of stress enzymes, such as peroxidase, ascorbate peroxidase, superoxide dismutase and catalase in pistil (Tsuruhara and Tezuka 2001, Neog *et al*. 2004). There are a few studies concerning changes in the activities of enzymes in stigma-style tissues following compatible and incompatible pollination. Since legitimate and illegitimate pollination are affected by alterations in the metabolic status of stigma and stylar tissues, it would be imperative to study the changes in the enzyme activities of stigma-style tissues following compatible and incompatible pollination. In present research, isoenzymes patterns and enzyme activity of guaiacol peroxidase, ascorbate peroxidase, superoxide dismutase and catalase in concerning H_2O_2 content (as a product and/or substrate for stress enzymes) were studied in two self-compatible and self-incompatible cultivars of *Petunia hybrida* following self- and cross-pollination.

MATERIALS AND METHODS

Seeds of two different cultivars of *Petunia hybrida,* e.g. Bravo purple star (self-incompatible) and Bravo cool water mix (self-compatible) were purchased from Syngenta seeds B.V. Company and grown in a greenhouse. For self-pollination, on the day of anthesis, the stigmas from each flower were hand-pollinated by pollen grains from the same flower. For cross-pollination, the flowers were emasculated and stigmas of each flower were pollinated by pollen grains from the other cultivar. To prevent natural pollination, petals were closed after hand pollination. After 48h, pollinated styles were collected and after fixation in liquid nitrogen preserved in -80°C until use.

Measurement of H_2O_2: H_2O_2 content was determined using the method given by Velikova et al. (2000). Style tissues were extracted with 5 ml of 0.1 % TCA (trichloroacetic acid) and centrifuged at 12000 *g* for 15 min. Then 0.5 ml of supernatant was mixed with 0.5 ml of 10 mM phosphate buffer (pH 7.0) and 1 ml of 1M potassium iodide and the absorbance was determined at 390 nm. The amount of H_2O_2 was read using the extinction coefficient 0.28μM^{-1} cm^{-1} and expressed as µmol g^{-1} fresh weight.

Extraction of enzyme: Frozen styles (0.5 g) was homogenized in 50 mM phosphate buffer (pH 7.2) containing 1 mM EDTA (ethylene diamine tetraacetic acid), 1 mM PMSF (phenylmethylsulphonyl fluoride) and 1% PVP (poly vinyl pyrrolidone). The homogenate was centrifuged at 14000g for 15 min at 4C. The supernatant was used directly for the assay of enzyme activity, isoenzyme patterns and estimation of protein. Enzymes extraction of sepal and petal were also prepared for studies of isoenzyme patterns as controls. Activity of enzymes was determined at 25°C with a UV-visible spectrophotometer (Varian Cary 50, USA). The supernatant was used to quantified total soluble protein according to Bradford (1976) and expressed in mg g^{-1} fresh weight. Bovine serum albumin was used as standard.

Assay of enzyme: Peroxidase (POD) activity was assayed by the method of Plewa *et al*. (1991) using the guaiacol test. Guaiacol oxidation (tetraguaiacol formation) was monitored by reading the absorbance at 470 nm at the moment of enzyme extract addition and 1 min later. The difference in absorbance (ΔA_{470}) was divided by the tetraguaiacol molar extinction coefficient (25.5 $mM^{-1}cm^{-1}$) and the enzyme activity was expressed as mmol of H_2O_2 $min^{>1}mg^{-1}$ of protein. The POD unit was calculated for the formation of 1 mM tetraguaiacol for 1 minute. Ascorbate peroxidase (APX) activity was measured using method of Nakano and Asada (1981). Decreasing absorbance at 290 nm considered as oxidation of ascorbic acid, was followed 3 min after starting the reaction. The difference in absorbance (ΔA_{290}) was divided by the ascorbate molar extinction coefficient (2.8 $mmol^{-1}cm^{-1}$). One unit of APX oxidizes 1mM ascorbic acid in one minute. Superoxide dismutase (SOD) activity was measured by monitoring the inhibition of NBT (nitroblue tetrazolioum) reduction at 560 nm (Giannopolitis and Ries 1977). The unit of SOD activity was defined as the amount of enzyme that inhibits the NBT photoreduction by 50 %. SOD activity values are given in units per mg of protein. Catalase (CAT) activity

was assayed using method of Dhindsa and Motowe (1981). The enzyme activity was estimated by monitoring the decrease in absorbance of H_2O_2 at 240 nm. Unit of activity was taken as the amount of enzyme which decomposes 1 mM of H_2O_2 in one minute.

Native PAGE and activity staining: Nondenaturating polyacrylamid gel electrophoresis (PAGE) was performed at 4 °C and 80 mA following Laemmli method (1970). Equal amounts of protein extracts were mixed with bromophenol blue and glycerol. For POD, the enzyme solutions were subjected to native PAGE with 10% polyacrylamide gel, for APX and SOD with 12% and for CAT with 7.5%.

POD isoenzymes were visualized by incubating of the gels in 25mM potassium phosphate buffer (pH 7.0) for 15 min. Then gels were submerged in a freshly prepared potassium phosphate buffer (pH 7.0) containing 18 mM guaiacol and 25 mM of H_2O_2. POD isoenzymes were appeared with brown bands after 10-30 min (Fielding and Hall 1978).

APX isoenzymes activity was detected by the procedure described by Mittler and Zilinskas (1993). The gel equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 2 mM ascorbate for 30 min, was incubated in the buffer for 1 min and submerged in a buffer solution consisting 28 mM TEMED (N,N,N',N'-tetramethylethylenediamine) and 2.45 mM NBT for 10-20 min with gentle agitation in the presence of light.

For SOD, the gel was stained according to the method of Rao *et al.* (1996). The native gels were incubated in 50 mM potassium phosphate buffer (pH 8.0) containing 5 mM H_2O_2 in the dark at 4 °C for 30 min After incubation the gels were immersed in buffer solution (pH 8.0) containing 2.5 mM NBT in the dark at 4 °C. After 25 min, gels submerged in buffer solution (pH 8.0) containing 28 mM riboflavin and 28 mM TEMED for 20 min in the dark at 4 °C. Then the gels were washed with distilled water and illuminated until the bands became apparent.

To visualize CAT profile, gels were stained by the procedure of Anderson *et al.* (1995). The gels were soaked in 3.27 mM H_2O_2 for 25 min, rinsed twice in distilled water, and stained in a freshly prepared solution containing 1% (w/v) potassium ferricyanide and 1% (w/v) ferric chloride.

Statistical analysis: Experiments related to enzyme activity and protein content was done with 3 replicates and all data were subjected to a one-way analysis of variance. Significant differences among individual means were determined using Duncan test and significance was determined at the 95 % confidence (P d" 0.05) limits.

RESULTS AND DISCUSSION

H_2O_2 content from styles of both cultivars was significantly higher in self-pollinated than cross-pollinated styles (Fig. 1A). The extent of increase in H_2O_2 content was more in self-pollinated styles in Bravo purple star cultivar. The previously reported results (Tezuka *et al.* 1997, Suzuki *et al.* 2001), together with our present results, suggest that self-incompatibility in *Petunia hybrida* might induce stress due to accumulation of free radicals in pistils upon self-incompatible pollination. Enzymes related to responses to stress appear to be activated and/or induced in pistils after self-incompatible pollination. This results in formation of large amount of active oxygen species such as H_2O_2. A stress response due to high levels of free radicals in pistils upon self-incompatible pollination could be induced after the recognition reaction. After that, the rejection reaction might be induced to inhibit the elongation of pollen tubes (Tezuka *et al.* 1997). Recently, H_2O_2 and other reactive oxygen species (ROS) have been shown to be involved in cell signaling in plants, where they regulate diverse aspects of plant metabolism and cell growth (Neill *et al.* 2002).

The highest activity of POD was found in self-pollinated Bravo purple star styles. There was no significant difference in POD activity of self- and cross-pollinated styles from Bravo cool water mix cultivar (Fig. 1B). POD enzyme may directly acts to prevent the growth of self-pollen tubes or sometimes may remain absent to disturb the growth of the pollen tube (Neog *et al.* 2004). McInnis *et al.* (2006) stated that higher activity of peroxidase as a major enzyme of angiosperm pistils, is related to higher H_2O_2 content. As well, peroxidases generally catalyze the breakdown of hydrogen peroxide

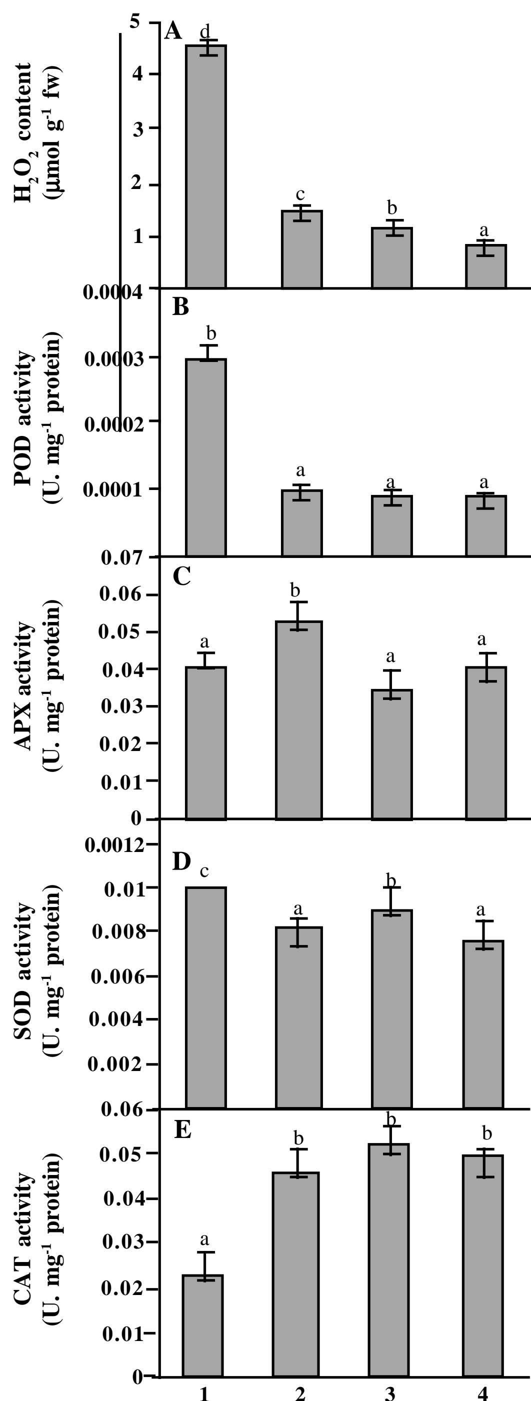


Fig. 1. Level of H_2O_2 content, activity of POD, APX, SOD and CAT (respectively graphs A, B, C, D and E) in the styles of *P. hybrida* Bravo purple star and *P. hybrida* Bravo cool water mix cultivar (1: self-pollinated Bravo purple star, 2: cross-pollinated, 3: self-pollinated Bravo cool water mix, 4-cross-pollinated Bravo cool water mix). Values are mean ± standard deviation of three set of samples.

to yield highly oxidizing intermediates, which oxidize a variety of organic and inorganic reducing substrates (Neog *et al*. 2004). Given that peroxidases can generate H_2O_2 as well as consume it (Bolwell *et al*. 2002). Isoenzyme studies in *Nicotiana* suggested a potential role for peroxidases in pollination process, particularly self-incompatibility. These studies showed the increased activity of stylar peroxidase in incompatible pollinations compared to compatible pollinations (Bredemeijer 1976). Carraro *et al*. (1986) suggested peroxidases are responsible for the inhibition of incompatible pollen tube growth in *Petunia* and *Nicotiana*. Isoenzymes pattern of POD did not show any difference between cross-compatible and self-incompatible pollination. These data possibly show that self-incompatibility could not affect gene(s) involving synthesis of new isoenzyme of POD.

The activity of APX was significantly lower in self-pollinated styles compared to cross-pollinated ones in Bravo purple star cultivar. In Bravo cool water mix cultivar there was no significant difference in APX activity of self- and cross-pollinated styles (Fig. 1C). The activity of SOD from both cultivars styles was promoted by self-pollination. The extent of the promotion was, however, more in self-pollinated flowers in Bravo purple star cultivar (Fig. 1D). The activity of CAT in the self-pollinated styles was also lower compared to cross-pollinated ones in Bravo purple star cultivar. The activity of catalase did not show any significant difference between self- and cross-pollinated styles of Bravo cool water mix cultivar (Fig. 1E). Findings of Suzuki *et al*. (2001) support our results. They reported that the activity of APX and CAT decreased in pistils with self-incompatible pollination compared to cross-compatible pollination in lily (gametophytic self-incompatible). According our results, higher activity of SOD was observed in self-pollinated styles of both cultivars but it showed higher extent in Bravo purple star cultivar. Within a cell, the superoxide dismutase (SOD) constitutes the first line of defense against ROS (Alscher *et al*. 2002). Van Camp *et al*. (1994) stated that over expression of SOD can lead to protection against specific stresses. Hydrogen peroxide is formed as a result of SOD action (Carraro *et al*. 1986, Takahashi and Asada 1983). It seems that high activity of SOD could be the main reason for high H_2O_2 content in self-pollinated styles of

Bravo purple star cultivar. APX, SOD and CAT isoenzymes did not show any changes between self- and cross-pollinated styles in both cultivars.

One POD and two CAT isoenzyme bands were detected in self- and cross-pollinated styles in both cultivars and there was no obvious variation in POD isoenzyme pattern between all samples (Fig. 2A, D). Three APX and SOD isoenzymes were found in self- and cross-pollinated styles in Bravo purple star cultivar while there were two isoenzymes in self- and cross-pollinated styles in Bravo cool water mix cultivar (Fig. 2B, C). Based on our results, it seems that self-pollination would not affect isoenzyme pattern of stress enzymes in the styles of *Petunia hybrida* Bravo purple star and *P. hybrida* Bravo cool water mix cultivars compared to cross-pollination. As the synthesis of these stress enzymes is controlled by their corresponding gene(s), the quantitative changes in their activities may be due to the changes in the regulation of expression of gene(s) and these changes in enzymes activity was not related to the expression of new isoenzymes of stress enzymes.

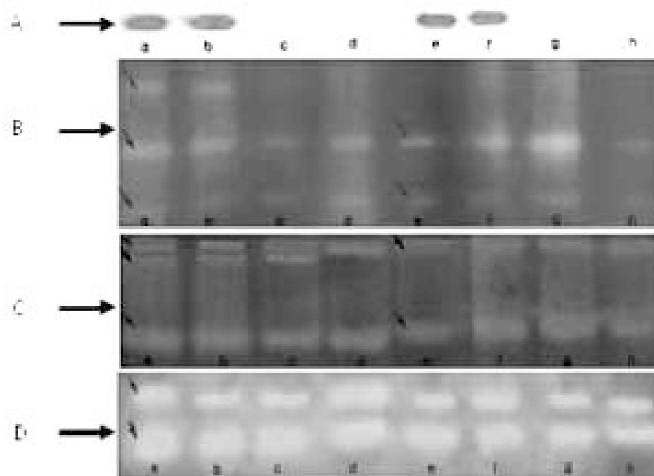


Fig. 2. Isoenzyme pattern of POD (A), APX (B), SOD (C) and CAT (D) in, a: self-pollinated styles, b: cross-pollinated styles, c and d: in turn sepal and petal in Bravo purple star cultivar, e: self-pollinated styles, f: cross-pollinated styles, g and h: in turn sepal and petal in Bravo cool water mix cultivar

As a whole, the results of present study similar to some other researches showed variation in the enzymes activity after self-incompatible pollination. Neog *et al.* (2004) stated that variations in the enzyme activities show that the biochemical changes occur in the stigma-style tissues affected by self-incompatibility mechanism that in turn affects the metabolic status of these tissues. Self-pollination would not affect isoenzyme pattern of stress enzymes in the styles of *Petunia hybrida* Bravo purple star and *P. hybrida* Bravo cool water mix cultivars compared to cross-pollination. The quantitative changes in the enzymes activities may be due to the changes in the regulation of expression of gene(s) and hence offer a basis to study the mechanism of self-incompatibility at molecular level.

REFERENCES

Alscher, R.G., Erturk, N. and Heath L.S. (2002). Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. *J. Exp. Bot.* **53:** 1331-1341.

Anderson, M.D., Prasad, T.K. and Stewart, C.R. (1995). Changes in isozyme profiles of catalase, peroxidase, and glutathione reductase during acclimation to chilling in mesocotyls of maize seedlings. *Plant Physiol.* **109:** 1247-1257.

Bolwell, G.P., Bindschedler, L.V., Blee, K.A, Butt, V.S., Davies, D.R., Gardner, S.L. and Minibayeva C.G. (2002). The apoplastic oxidative burst in response to biotic stress in plants: A three component system. *J. Exp. Bot*. **53:** 1367-1376.

Bradford, M.M. (1976). A rapid and sensitive for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248-254.

Bredemeijer, G.M.M. (1976). Effect of bud-pollination and delayedself-pollination on the induction of possible rejection peroxidase in styles of *Nicotiana alata*. *Acta Bot. Neerl.* **25:** 107-116.

Carraro, L., Lombardo, G. and Gerola, F.M. (1986). Stylar peroxidase and self-incompatibility reactions in *Petunia hybrida. J. Cell Sci*. **82:** 1-10.

Dhindsa, R.S. and Motowe, W. (1981). Drought tolerance in two mosses: correlation with enzymatic defense against lipid peroxidation. *J. Exp. Bot.* **32:** 79-91.

Fielding, J.L. and Hall, J.L. (1978). A biochemical and cytological study of peroxidase activity in root of *Pisum sativum*. *J. Exp. Bot.* **29:** 968-981.

Giannopolitis, C.N. and Ries, S.K. (1977). Superoxide dismutase occurrence in higher plants. *Plant Physiol.* **59:** 309-314.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227:** 680-685.

McClure, B. (2006). New views of S-RNase-based self-incompatibility. *Cur. Opinion in Plant Biol.* **9:** 639-646.

McCormic, S. (1998). Self-incompatibility and other pollen-pistil interactions. *Cur. Opinion in Plant Biol.* **1:** 18-25.

McInnis, S.M., Emery, D.C., Porter, R., Desikan, R., Hancock, J.T. and Hiscock, S.J. (2006). The role of stigma peroxidases in flowering plants: insights from further characterization of a stigma-specific peroxidase (SSP) from *Senecio squalidus* (Asteraceae). *J. Exp. Bot.* **57:** 1835-1846.

Mittler, R. and Zilinskas, B. (1993). A detection of ascorbate peroxidase activity in native gels by inhibition of the ascorbate-dependent reduction of nitroblue tetrazolium. *Anal Biochem.* **212:** 540-546.

Nakano, Y. and Asada, K. (1981). Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant Cell Physiol.* **22:** 867-880.

Neill, S., Desikan, R. and Hancock, J. (2002). Hydrogen peroxide signalling. *Cur. Opinion in Plant Biol.* **5:** 388-395.

Neog, B., Yadav, R.N.S. and Singh, I.D. (2004). Peroxidase, polyphenol oxidase and acid phosphatase activities in the stigma-style tissue of *Camellia sinensis* (L) O. Kuntze following compatible and incompatible pollination. *J. Indian Inst. Sci.* **84:** 47-52.

Plewa, M.J., Smith, S.R. and Wagner, E.D. (1991). Diethyldithiocarbamate suppresses the plant activation of aromatic amines into mutagens by inhibiting tobacco cell peroxidase. *Mutation Res.* **247:** 57-64.

Rao, M.V., Paliyath, G. and Ormrod, D.P. (1996). Ultraviolet-B- and ozone-induced biochemical changes in antioxidant enzymes of *Arabidopsis thaliana. Plant Physiol.* **110:** 125-136.

Roalson, E.H. and McCubbin, A.G. (2003). S-Rnases and sexual incompatibility: structure, functions, and evolutionary perspectives: *Mol. Phylogenet. Evol.* **29:** 490-506.

Rudd, J.J. and Franklin-Tong, V. (2003). Signals and target of the self-incompatibility response in pollen of Papaver rhoeas. *J. Exp. Bot.* **54:** 141-148.

Shivanna, K.R. and Sharma, N. (1976). Self-incompatibility recognition in *Petunia hybrida. Micron and Microscopica Acta.* **16:** 233-245.

Suzuki, H., Tsuruhara, A. and Tezuka, T. (2001). Regulations of the C_2H_4-forming system and the H_2O_2-scavenging system by heat treatment associated with self incompatibility in lily. *Sex Plant Reprod.* **13:** 201-208.

Takahashi, M. and Asada, K. (1983). Superoxide anion permeability of phospholipid membranes and chloroplast thylakoids. *Arch. Biochem. Biophys.* **226:** 558-566.

Tezuka, T., Tsuruhara, A., Suzuki, H. and Takahashi, S.Y. (1997). A connection between the self-incompatibility mechanism and the stress response in lily. *Plant Cell Physiol.* **38:** 107-112.

Thompson, R.D. and Kirch, H.H. (1992). The S locus of flowering plants: when self-rejection is self-interest. *Trends Genet.* **8:** 381-387.

Tomimoto, Y., Nakazaki, H., Ueno, H. and Hayashi, R. (1996). Analysis of self-incompatibility-related ribonucleases (S-RNases) in two species of pears, *Pyrus communis* and *Pyrus ussuriensis. Scientia Hortic.* **66:** 159-157.

Tsuruhara, A. and Tezuka, T. (2001). Relationship between the self-incompatibility and cAMP level in *Lilium longiflorun. Plant Cell Physiol.* **42:** 1234-1238.

Van Camp, W.H., Willekens, C., Bowler, M., Van Montagu, D., Langebartels, I.C. and Sandermann, H. (1994). Elevated levels of superoxide dismutase protect transgenic plants against ozone damage. *Biol. Tech.* **12:** 65-8.

Velikova, V., Yordanov, I. and Edreva, A. (2000). Oxidative stress and some antioxidant systems in acid rain-treated bean plants. *Plant Sci.* **151:** 59-66.

Wang, Y., Wang, X., Skirpan, A.L. and Kao, T.H. (2003). S-RNase mediated self-incompatibility. *J. Exp. Bot.* **54:** 115-122.s