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ORIGINAL ARTICLE

Sodium nitroprusside mediates seedling development and attenuation of oxidative stresses in Chinese cabbage

Chang Hyun Sung · Jeum Kyu Hong

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Abstract Nitric oxide (NO) has been shown to be involved in diverse physiological processes in microbes, animals and plants. In this study, the involvement of NO in the development and possible roles in oxidative stress protection of Chinese cabbage (*Brassica rapa* subsp. *pekinensis* cv. Samrack-ulgari) seedlings were investigated. Exogenous application of sodium nitroprusside (SNP) retarded root elongation, while increasing lateral root formation of Chinese cabbage. Plants showed no signs of external stress due to SNP application in true leaves. Cotyledons of 3-week-old Chinese cabbage plants were found to be highly sensitive to SNP application. Treated cotyledons displayed rapid tissue collapse and associated cell death. Although SNP application reduced root growth under normal growth conditions, it also enhanced methyl viologen (MV)-mediated oxidative stress tolerance. Analysis of SNP application to Chinese cabbage leaf disks, revealed SNP-induced tolerance against oxidative stresses by MV and H_2O_2, and evidence includes prevention of chlorophyll loss, superoxide anion (O_2^-) accumulation and lipid peroxidation. This report supports a role for nitric oxide in modulating early seedling development, programmed cell death and stress tolerance in Chinese cabbage.

Keywords Chinese cabbage · Nitric oxide · Oxidative stress · Root development

C. H. Sung · J. K. Hong (✉)
Laboratory of Plant Pathology and Protection,
Department of Horticultural Sciences,
College of Life Sciences and Natural Resources,
Jinju National University, 150 Chilam-dong, Jinju,
Kyungnam 660-758, Republic of Korea
e-mail: jkhong@jinju.ac.kr

Abbreviations

DAB	3,3′-Diaminobenzidine
MV	Methyl viologen
NBT	Nitro blue tetrazolium
NO	Nitric oxide
PCD	Programmed cell death
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SNP	Sodium nitroprusside

Introduction

Multiple signaling pathways are involved in plant growth and development including processes such as seed germination and dormancy (Finkelstein et al. 2008; Seo et al. 2009), stomata formation (Nadeau 2009), root organogenesis (Fukaki and Tasaka 2009), and leaf senescence (Lim et al. 2007). Endogenous and exogenous stimuli in plants trigger certain cellular processes crucial for developmental progression. Certain plant hormones such as auxin and cytokinin participate in a diversity of physiological processes.

Plants defend themselves against biotic and abiotic stresses with highly sophisticated molecular mechanisms. A variety of defense signaling pathways are triggered by external stimuli with concomitant induction of defense-related gene expression (van Loon et al. 2006). Salicylic acid (SA)-, jasmonic acid (JA)- and ethylene-mediated defense signal induction pathways have been well characterized in plants (Dong 1998). Recent studies have demonstrated that abscisic acid (ABA) is also closely related to disease resistance as well as to environmental stress

tolerance (Seki et al. 2007; Ton et al. 2009). Genetic approaches using various Arabidopsis mutants defective in defense regulatory gene function have provided insights into molecular aspects of plant defense signaling (Dong 1998; Seki et al. 2007).

In recent years, there has been increasing evidence that NO functions as a diffusible signaling mediator in plant development and defense in response to pathogen infections. NO have been participated in diverse physiological processes in many plants, such as seed germination (Sarath et al. 2006), root organogenesis (Pagnussat et al. 2002), defense responses to biotic and abiotic stresses (Delledonne et al. 1998; Martin et al. 2009; Zhao et al. 2007). In terms of plant defenses, NO production is induced by biotic and abiotic stresses in plants (Ahlfors et al. 2009; Piterková et al. 2009). Application of plants with NO led to enhanced plant tolerance to pathogen infections and environmental stresses, while NO scavengers or biosynthesis inhibitors compromised the observed stress tolerance. Many forms of NO donors have been used for NO treatment, releasing gaseous NO, which easily diffuses throughout cells and tissues of the recipient organism. Despite current data, there is no convincing evidence of NO biosynthetic pathways and NO-associated biochemical reactions occurring in plants. In contrast, NO metabolism is well characterized in animals (Mayer and Hemmens 1997; Wendehenne et al. 2001). In animals, three isoforms of nitric oxide synthase (NOS) (iNOS, nNOS and eNOS) played roles in the immune, nervous (Dolan et al. 2003) and cardiovascular systems (Huang 2009) at different cellular locations (Mayer and Hemmens 1997). In *Arabidopsis*, nitric oxide-associated 1 (NOA1) protein (Guo et al. 2003) and two nitrate reductase isoforms (Desikan et al. 2002) were suggested as molecular mediators for NO signaling (Besson-Bard et al. 2008). Several hypotheses on NO action in plant tissues during defense responses have been suggested: (1) reactive oxygen species (ROS) produced during the defense responses to biotic and abiotic stresses interacted with NO regulating disease resistance and programmed cell death (PCD) processes; (2) NO triggers many kinds of redox-regulated defense-related gene expression directly or indirectly to establish plant stress tolerance (Parani et al. 2004; Polverari et al. 2003); and (3) basal disease resistance and hypersensitive response (HR) signaling could be modulated by post-translational modification of target functional proteins by NO, so-called protein *S*-nitrosylation (Feechan et al. 2005; Romero-Puertas et al. 2008).

Chinese cabbage (*Brassica rapa* subsp. *pekinensis*) is one of the most economically important vegetables in Asia. Environmentally friendly agricultural practises are needed for stable crop production. To date, the involvement of bioactive NO signal molecules in the physiology of Chinese cabbage has not been investigated. In this paper, we have investigated the responses of Chinese cabbage to SNP application (NO donor compound) particularly focusing on seedling development and oxidative stress protection.

Materials and methods

Plant growth

Chinese cabbage (*Brassica rapa* subsp. *pekinensis* cv. Samrack-ulgari) seeds were sterilized with 1% sodium hypochlorite (NaOCl) for 10 min and then washed 5 times with sterilized water. Seeds were transplanted for the root growth assay in different Murashige–Skoog (MS) agar media. The MS agar plates were placed vertically during root elongation. For experiments using Chinese cabbage leaves and cotyledons, the seeds were imbibed on water-saturated filter paper for 1 day, and then sown in soil mixture. The Chinese cabbage plants were raised in a growth room under the controlled environments of 23 ± 2°C and 70 μmol photons m^{-2} s^{-1} illumination with 12 h light/12 h dark photoperiod at 60% relative humidity.

Chemical treatments

SNP was used as the donor molecule for the nitric oxide (NO) production. To evaluate response of early seedling growth to exogenous SNP, Chinese cabbage seeds were germinated and, 1 day after germination, were then transferred to MS agar media supplemented with different concentrations of SNP (5, 10, 25, 50, 100, 250, and 500 μM). Different concentrations of SNP solutions (0.1, 0.5, 1, and 5 mM) were foliar sprayed onto 3-week-old Chinese cabbage seedlings growing in a soil mixture to evaluate the cellular response induced in cotyledons and true leaves.

Different growing stages of Chinese cabbage seedlings/plantlets were treated with MV, a herbicide that elevates the cellular ROS as a oxidative stress-inducing agent. For root growth evaluation, increasing doses of MV were added in combination with SNP into MS media. To investigate the protective effect of SNP application in true leaves, Chinese cabbage seedlings were sprayed with different concentrations of SNP, and then 100 μM of MV was sprayed 24 h after SNP treatment.

For leaf disk assays, Chinese cabbage leaf disks (ϕ 10 mm) were floated onto solution containing different concentrations of SNP and/or MV. H_2O_2 was also used for oxidative stress-inducing agent in the leaf disk assay.

Chlorophyll content

The chlorophyll content of Chinese cabbage leaf disks was measured after SNP and/or MV, and SNP and/or H_2O_2

treatments. The chlorophyll in the leaf disks were extracted with 95% ethanol at 50°C for 3 h, and the content was determined spectrophotometrically based on the formula $\mathrm{Chl}(A + B) = 5.24\ A_{664} + 22.24\ A_{648}$, where Chl is the chlorophyll concentration in microgram per milliliter and A is the absorption (Lichtenthaler 1987).

Histochemical staining

Cellular damage in cotyledons was detected using Evans blue staining. Plant tissues were stained with 0.5% Evans blue for 1 h. After washing with distilled water several times, cotyledons were photographed. For quantitative analysis, leaf disks (ϕ10 mm) from Evans blue-stained cotyledons were destained with 50% methanol and 1% SDS for 50°C for 1 h and stain uptake was quantified spectrophotometrically at 565 nm (Martin et al. 2009; Oh et al. 1999). Determinations were performed at least 3 times with 4 leaf disks each.

Stress-induced generation of O_2^- in situ was detected by nitro blue tetrazolium (NBT) staining. 0.1% of NBT in 10 mM sodium azide (NaN_3) until a purple-blue color became visible (Lin et al. 2009), and the chlorophyll of the treated sample was removed with 95% ethanol. H_2O_2 production was detected by 3,3′-diaminobenzidine (DAB) staining as described by Thordal-Christensen et al. (1997). Leaf disks were stained 0.1% DAB solution for 6 h, and then chlorophyll was removed with 95% ethanol. The decolorized leaf disks were photographed.

Leaf disks were stained with Schiff's reagent for stress-induced lipid peroxidation (Yamamoto et al. 2001). Leaf disks were stained with Schiff's reagent (Sigma) for 30 min, and rinsed with 0.5% (w/v) $K_2S_2O_5$ in 0.05 M HCl, which detects aldehydes originated from lipid peroxides.

Results

NO-mediated growth change during early root and cotyledon development

Chinese cabbage seedlings were grown on MS media containing increasing concentration of SNP. Root growth in response to exogenous SNP was examined 2 and 6 days after transfer to SNP-supplemented MS media (Fig. 1). A concentration of less than 10 μM affected slightly primary root elongation of Chinese cabbage 2 days after treatment, but the growth limit was released 6 days after treatment. Concentrations in the range from 25 to 500 μM reduced root elongation 2–6 days after treatments (Fig. 1b). SNP treatment also affected lateral root formation (Fig. 1c). An SNP concentration of 25–100 μM was sufficient for increased formation of lateral roots, but a much higher concentration of SNP significantly decreases the number of lateral roots, probably because of reduced root elongation. High concentration of SNP treatment also arrested cotyledon development indicated by decreased fresh weight (Fig. 1d). Higher than 25 μM SNP reduced fresh weight of cotyledons at 2 days after treatment, but significant arrested cotyledon developments were mediated by above 50 μM SNP at 6 days after treatment.

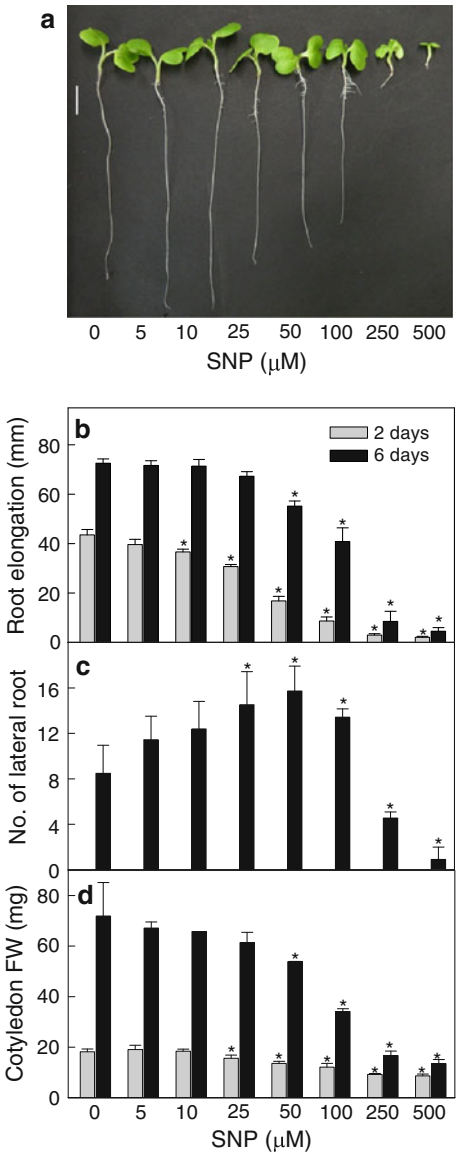

Fig. 1 SNP-mediated early seedling development in Chinese cabbage. **a** SNP-responsive seedling growth. Photo was taken 6 days after SNP treatment. *Bar* 1 cm. **b** Primary root elongation inhibition, **c** lateral root formation and **d** cotyledon development of the seedlings in response to different concentrations of SNP treatment at 2 and 6 days. Data are the means ± standard errors from four independent experiments performed with similar results ($n = 8$). *Asterisks* above the bars indicate significantly different root growth compared to seedlings grown on normal growth condition without SNP at $P < 0.05$ (t test)

NO-induced cellular damage

Treatment of 3-week-old Chinese cabbage seedlings with increasing concentrations of SNP resulted in cellular damage to cotyledons (Fig. 2). Foliar spray of 5 mM SNP caused severe cellular damages of cotyledons (Fig. 2a). Cotyledons started to wilt within 6 h after 5 mM SNP treatment and finally resulted in death. By contrast, true leaves were not significantly damaged after SNP spraying. Cotyledons floating on increasing concentrations of SNP solutions showed water-soaking related damage 2 days after treatment (Fig. 2b). Cellular damages could also be observed by Evans blue staining (Fig. 2c). Dark blue stains were detected in severely affected cotyledons due to higher SNP concentrations, and quantification of stained cotyledons was performed as shown in Fig. 2d.

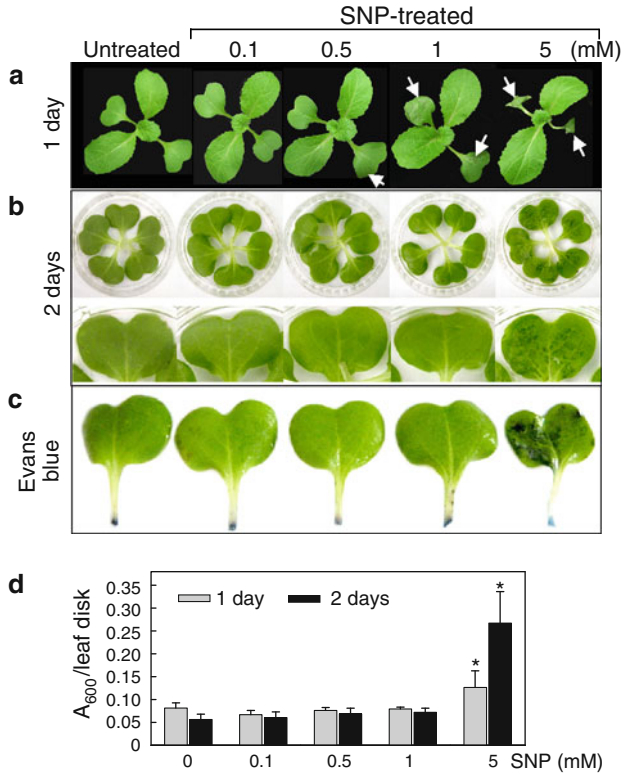

Fig. 2 SNP-mediated cellular damages in Chinese cabbage. **a** Cellular damage of cotyledons by spraying with different concentrations of SNP at 24 h after treatment. *Arrows* indicate symptomatic areas with collapsed tissues. **b** Water-soaked symptom of cotyledons floated onto different concentrations of SNP solution 48 h after treatment. *Upper* detached cotyledons treated with different concentration of SNP solution. *Bottom* magnified cotyledons showing representative symptom. **c** Cotyledons stained with Evans blue 48 h after SNP treatment. **d** Quantitative measurement of Evans blue uptake in cotyledons after SNP treatment spectrophotometrically. Data are the means ± standard errors from four independent experiments performed with similar results ($n = 8$). *Asterisks* above the bars indicate significantly different root growth compared to seedlings grown on normal growth condition at $P < 0.05$ (t test)

Role of NO against MV-induced oxidative stress during early root and cotyledon development

One day after seed germination in sterilized water, germinated Chinese cabbage seeds were transferred onto MS supplemented with different concentrations of SNP and/or MV. Relative primary root elongation was investigated 3 days after transferring to SNP and/or MV (Fig. 3a,

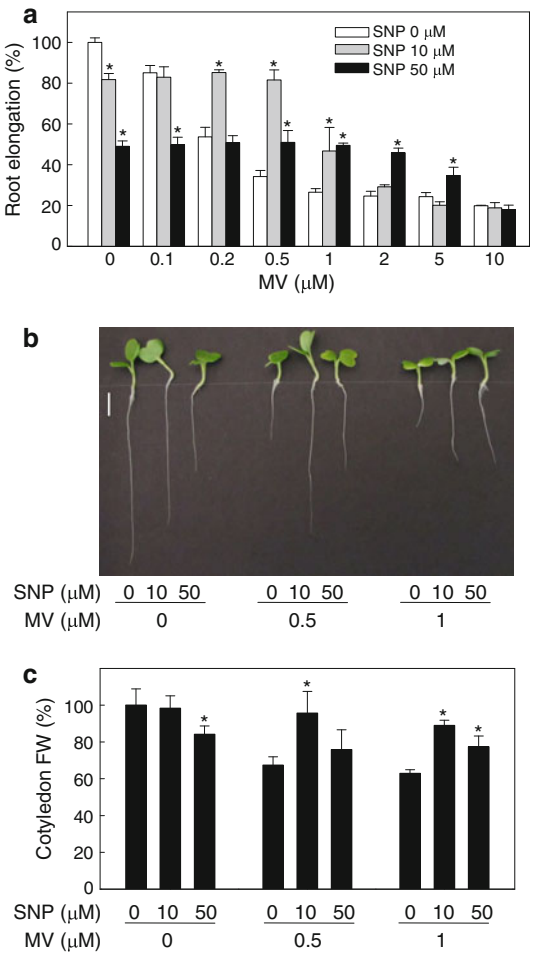

Fig. 3 Protective effect of SNP on MV-mediated root elongation inhibition of Chinese cabbage seedlings. Germinated seeds were transferred on MS media containing SNP and/or MV, and root growth was measured 3 days after treatment. **a** Relative primary root elongation of the seedlings in response to SNP and/or MV treatment. Data are the means ± standard errors from three independent experiments performed with similar results ($n = 5$). *Asterisks* above the bars indicated significantly different value compared to SNP-untreated cotyledons at $P < 0.05$ (t test). **b** Photo was taken 3 days after SNP and/or MV treatment. *Bar* 1 cm. **c** Relative cotyledon fresh weight (FW) of the seedlings treated with SNP and/or MV measured 3 days after SNP and/or MV treatment. Data are the means ± standard errors from three independent experiments performed with similar results ($n = 5$). *Asterisks* above the bars indicate significantly different value compared to SNP-untreated cotyledons at $P < 0.05$ (t test)

b). SNP treatment led to reduced root elongation, demonstrating that 10 and 50 μM SNP retarded root growth by 81.8 and 49.0%, respectively, compared to root growth without any stress treatment. Also, 0.1 μM of MV decreased root growth without SNP, but root elongation was not arrested in the presence of 10–50 μM SNP. Treatment with 0.2 μM of MV drastically reduced root growth to 53.7%, which is similar to the degree of root growth retardation caused by treatment with high concentrations of SNP. Although root elongation decreased to 34.2% when 0.5 μM of MV was applied in the absence of SNP, root growth remained unchanged with SNP application, indicating a protective effect of SNP against MV stress. When the MV concentration was increased to 2–5 μM, 10 μM of SNP was insufficient for protection. Treatment with 10 and 50 μM SNP was not able to prevent oxidative stress caused by the application of 10 μM of MV. Cotyledon development was also evaluated during significant root protection by SNP against MV stress (Fig. 3b, c) as demonstrated in Fig. 3a. SNP at 50 μM slightly affected cotyledon development without MV stress. Treatment with 10 μM of SNP efficiently released cotyledon development arrest by 0.5 and 1 μM of MV stresses; however, 50 μM of SNP could not protect cotyledons against 0.5 μM of MV stress.

NO against MV- and H_2O_2-induced oxidative stress in leaf tissues

Pretreatment of SNP was enough to induce enhanced tolerance to oxidative stress in Chinese cabbage (Fig. 4). SNP was foliar-sprayed onto 3-week-old Chinese cabbage seedlings, and after 1 day was followed by an MV treatment. Seedlings were severely damaged 2 days after MV treatment. True leaves and cotyledons had collapsed and wilted (Fig. 4a). However, increasing the dose of SNP resulted in protection of true leaves against oxidative stress by MV being observed. At 3 days after SNP treatment without MV, Chinese cabbage seedlings showed similar responses to those observed 1 day after SNP treatment (Fig. 2a).

Leaf disks were floated on solutions containing various combinations of SNP and MV concentrations (Fig. 4b). MV-induced stress was shown by bleaching of leaf disks, indicating loss of chlorophyll 3 days after treatment. However, treatment with SNP protected leaf disks when MV was applied. Loss of chlorophyll was measured and demonstrated in Fig. 4c. Without MV stress, no difference was found in the chlorophyll content in leaf disks regardless of SNP treatment. Although MV caused drastic chlorophyll loss and bleaching in leaf disks, leaf disks in the presence of SNP have relatively higher chlorophyll contents.

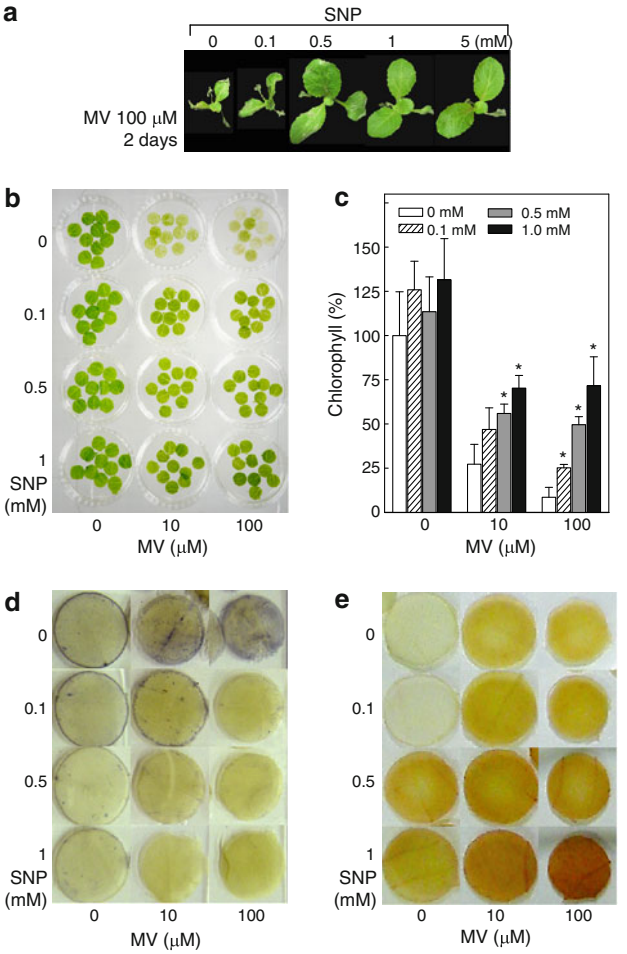

Fig. 4 SNP-mediated protection in Chinese cabbage true leaves against MV-induced oxidative stress. **a** Enhanced tolerance to MV treatment by foliar spray of SNP. At 1 day after SNP pre-treatment, MV was sprayed and photo was taken 2 days after MV treatment. **b** Induced tolerance of leaf disks to MV treatment by SNP. Ten leaf disks were floated onto different concentration of SNP and/or MV. Photograph was taken 3 days after floating. **c** Relative chlorophyll content of leaf disks floated on MV solution in the absence or presence of SNP. Data are the means ± standard errors from three independent experiments performed with similar results ($n = 5$). *Asterisks* above the bars indicated significantly different chlorophyll contents compared to SNP-untreated cotyledons at $P < 0.05$ (t test). **d** Histochemical accumulation of superoxide anion (O_2^-) detected by NBT staining of leaf disks floated on MV solution in the absence or presence of SNP. **e** Histochemical accumulation of H_2O_2 detected by DAB staining of leaf disks floated on MV solution in the absence or presence of SNP

MV leads to oxidative stress, generating superoxide anion (O_2^-) in a light-dependent manner. Leaf disks with differential responses to MV treatment due to SNP addition were stained with NBT to detect O_2^- accumulation (Fig. 4d). Leaf disks with no SNP and no MV only showed purple-blue color at the margin of the disks. The staining color gradually disappeared by increasing the concentration of SNP used. Without SNP treatment, whole areas of

leaf disk were stained with NBT because of MV-mediated oxidative stress. By contrast, SNP application compromised the staining under MV treatment. Purple-blue color precipitates were observed in enlarged photos of leaf areas under MV-stressed condition without SNP (data not shown). Because O_2^- is usually rapidly converted into H_2O_2 and an oxygen molecule (O_2) by superoxide dismutase (SOD) enzymatically, we investigated H_2O_2 accumulation in MV-stressed leaf disks by DAB staining method (Fig. 4e). H_2O_2 was not detected in untreated leaf disks, and treatment with MV increased H_2O_2 accumulation. Interestingly, SNP alone led to moderate levels of H_2O_2 production in leaf disks without cellular damage and chlorophyll breakdown. However, increasing SNP under MV stress surprisingly, and perhaps synergistically, induced higher H_2O_2 production, when leaf disks displayed high chlorophyll contents with no cellular damage.

Excessive H_2O_2 treatment has resulted in similar physiological 'loss of chlorophyll' phenotype observed in leaf disks under MV stress. SNP treatment provided efficient protection of the chlorophyll of leaf disks exposed to H_2O_2 -mediated stress (Fig. 5).

NO can delay MV- and H_2O_2-induced lipid peroxidation in the plants

We investigated the involvement of NO in lipid peroxidation of Chinese cabbage during oxidative stress. Leaf disks under MV- and H_2O_2-induced oxidative stresses were stained with Schiff's reagent to detect histochemical lipid peroxidation (Fig. 6). Highly damaged leaf disks revealed dark pink color stains, whereas leaf disks showing SNP-mediated enhanced stress tolerance were less darkly stained.

Discussion

We investigated seedling responses of Chinese cabbage to exogenous SNP treatment. Many plant species including tobacco produce NO during root growth, but there is still limited information on the source and the role of NO per se in plants including root tissues (Stöhr and Stremlau 2006; Stöhr and Ullrich 2002). Reduced primary root elongation and induced lateral root emergence of Chinese cabbage by SNP treatment observed in this study were similar to the findings revealed in tomato plants (Correa-Aragunde et al. 2004). However, maize root elongation was promoted by treatment with very low concentration (10^{-10} M) of SNP (Gouvéa et al. 1997). These findings indicate that NO functions in root growth elongation differently in a dose-dependent manner, meaning that maintaining critical concentration of endogenous NO is crucial for homeostasis of different cellular systems. Different hormones were suggested as signaling molecules for lateral root formation in plants (Fukaki and Tasaka 2009). A role of NO during lateral root formation is a novel suggestion. Carbon monoxide (CO)-induced lateral root initiation was mediated by auxin and NO in tomato (Guo et al. 2008). Increased lateral root formation by NO was closely related to the role of auxin and expression of cell cycle regulatory genes like *CYCD3;1* and *KRP2* in tomato root (Correa-Aragunde et al. 2006). Kolbert et al. (2008) investigated the involvement of NO signaling in auxin-mediated lateral root

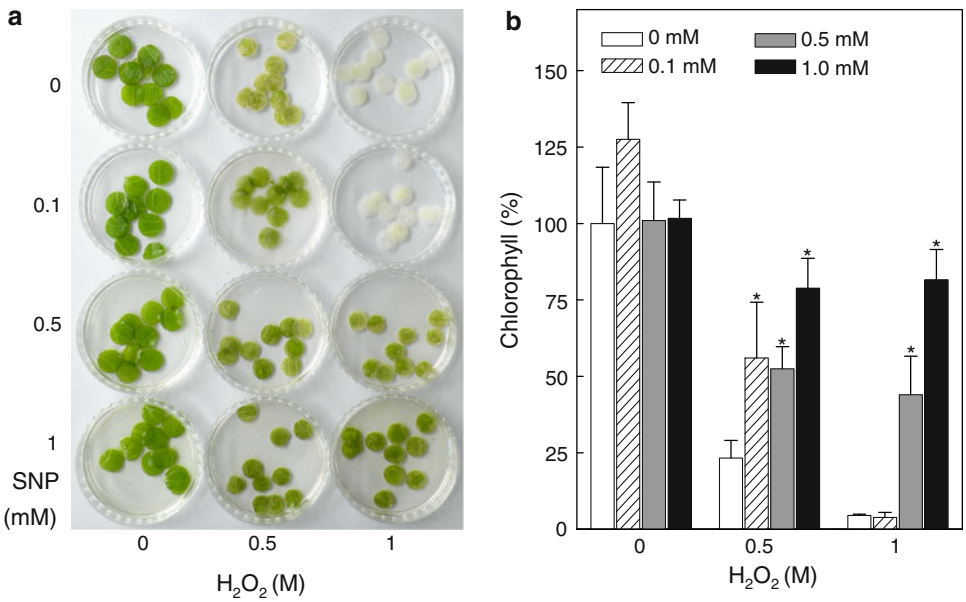

Fig. 5 SNP-mediated protection against H_2O_2-mediated oxidative stress in Chinese cabbage. **a** Chinese cabbage leaf disks floated on different concentration of SNP and H_2O_2 5 days after treatment. **b** Relative chlorophyll content of leaf disks floated on H_2O_2 solution in the absence or presence of SNP. Data are the means ± standard errors from three independent experiments performed with similar results ($n = 5$). *Asterisks* above the bars indicate significantly different chlorophyll contents compared to SNP-untreated cotyledons at $P < 0.05$ (t test)

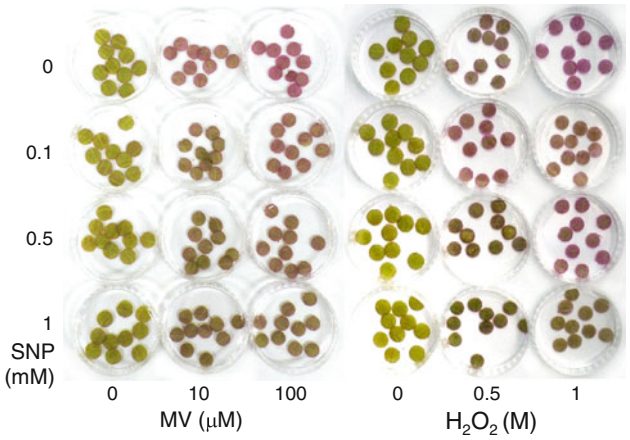

Fig. 6 SNP-mediated protection of lipid peroxidation in Chinese cabbage leaves against oxidative stress. Schiff's reagent staining of MV- and H_2O_2-induced leaf disks 3 and 5 days after treatment, respectively

formation in *Arabidopsis*. Genetic and pharmaceutical analyses suggested that auxin-induced lateral root formation required *NIA1* and *NIA2*, but not *NOA1*, which encode nitrate reductase 1 and 2, and nitric oxide associated 1 protein, respectively. During root elongation of Chinese cabbage (*Brassica chinensis*), NO accumulation was detected microscopically under normal growth conditions, and higher NO concentrations than that produced by SNP treatment (Du et al. 2008). Low levels of NO may be required for normal root growth of Chinese cabbage, but excessive levels of NO accumulation can cause adverse effects on root elongation as shown in this study. Treatment with NAA, synthetic auxin, also increases NO accumulation in Chinese root tissues (Du et al. 2008), indicating support for involvement of NO and auxin in root growth retardation and enhanced lateral root formation following SNP treatment. A role of NO in auxin signaling in Chinese cabbage root development requires further study. Cotyledon development of Chinese cabbage seedlings was also affected by the high concentration of SNP treatment in this study. NO inhibited hypocotyls elongation in lettuce plants (Beligni and Lamattina 2000). However, little effect was demonstrated on the physiological involvement of NO in cotyledon development in early seedling growing stage. It is worth noting that NO is related to root elongation, as well as cotyledon development in Chinese cabbage.

NO tightly regulates PCD together with ROS accumulation in plants to enhance disease resistance (Delledonne et al. 1998, 2001). Cell death was accelerated in cultured cell suspension of soybean by a relatively low concentration (0.5 mM) of SNP treatment, whereas high SNP (20 mM) diminished cell death. Additional ROS treatment with this high concentration of SNP triggered cell death (Delledonne et al. 2001). Foliar spray of 5 mM of SNP was sufficient for triggering drastic cellular damages in cotyledons, not true leaves, without additional stress at the early stage of Chinese cabbage seedling growth in our study. It may be due to differences in SNP-induced NO production efficiency in different plant systems, supported by the fact that mature rosette leaves of *Arabidopsis* infiltrated with 5 mM SNP underwent dramatic tissue collapse in continuous light conditions within 1 week (Murgia et al. 2004). Interestingly, cotyledons showed greater sensitivity than true leaves of Chinese cabbage seedlings in response to SNP treatment. It appears that different cellular redox conditions are likely to be present in cotyledons compared to true leaves, thus leading to differing sensitivity against exogenous NO application. Involvement of redox-regulated genes and enzymes in NO-responsiveness of Chinese cabbage clearly requires further investigation.

The role of NO in relation to plant defense to biotic and abiotic stresses is an intriguing question (Beligni and Lamattina 1999a). NO plays a role as a cytotoxic or cytoprotective agent in diverse physiological processes in plants which implies that cellular redox conditions play a major part in stress response effects. Exogenous NO is able to attenuate ROS-mediated oxidative stresses in potato leaves (Beligni and Lamattina 1999b), cucumber and rice roots (Shi et al. 2007; Singh et al. 2009), and *Arabidopsis* seedlings (Zhao et al. 2007). In Chinese cabbage, SNP-induced alleviation of MV-mediated oxidative stress has been observed during root and seedling development in the current investigation. SNP treatment resulted in different effects on root elongation under normal growth conditions and under oxidative stress conditions. Root growth and cotyledon development was arrested by SNP treatment without oxidative stress in a dose-dependent manner, while SNP promoted root elongation and cotyledon growth under stress conditions compared to non-stressed plantlets. Our results are similar to those observed for *Hibiscus moscheutos* root elongation inhibition by aluminum (Tian et al. 2007), suggesting that endogenous NO level is critical in regulating root elongation under normal and stress conditions. In this study, it is worth noting that SNP could also protect cotyledon growth during Chinese cabbage seedling development limited by MV stress.

Severe injuries caused by MV and H_2O_2 were observed by chlorophyll breakdown. In contrast, SNP-induced alleviation of leaf disks exposed to MV stress was evidenced by chlorophyll retention and reduced lipid peroxidation. Chloroplasts are known as vulnerable and sensitive cellular organelles, being shown to respond to various external stimuli in plant cells (Doyle et al. 2010; Li et al. 2006), as well as mediating stress signal communication with the nucleus in order to regulate PCD (Doyle et al. 2010; Galvez-Valdivieso and Mullineaux 2010). Treatment of barley seedlings with SNP increased chlorophyll content

and thylakoid membrane protein expression (Zhang et al. 2006). Chloroplast disorganization and suppression of chloroplast-encoding mRNAs in iron-deficient maize leaves was reversed by SNP supplementation (Graziano et al. 2002). NO may protect leaf tissues against oxidative stresses in Chinese cabbage through chloroplast modification.

Cellular membranes contain lipid components essential for living organisms. Under stress conditions, H_2O_2 and O_2^- can be converted into highly reactive oxidant OH· causing lipid peroxidation (Aurand et al. 1977), which is commonly used as a major index of plant environmental stresses. Lipid radicals and reactive aldehydes produced by lipid peroxidation cause disruption of the membrane lipid bilayer and membrane proteins (Gonçalves et al. 2009). In the present study, lipid peroxidation was detected histochemically in highly stressed leaf disks, but relatively lower lipid peroxidation occurred in leaf disks protected by SNP treatment. These indicate that NO interact with O_2^-, H_2O_2, as well as with OH·, in Chinese cabbage to mitigate ROS-mediated oxidative stresses.

SNP treatment alone did not cause any visible changes in leaf disks of Chinese cabbages. However, leaf disks under a no stress condition was thought to produce O_2^- at the wounded margin, but SNP compromised O_2^- accumulation, indicating that NO can be involved in wound healing process as suggested by París et al. (2007).

H_2O_2 might be closely related to SNP-induced tolerance to MV stress in Chinese cabbage. In general, plant tissues under various environmental stresses produce large amounts of H_2O_2. Although a high dosage of exogenous H_2O_2 application results in drastic cellular damage, H_2O_2 produced during SNP-induced MV stress tolerance was likely involved in the protection of leaf disks. H_2O_2 accumulation in highly damaged leaf disks by MV could be the result of O_2^- degradation by SOD. However, MV-induced cellular damage was not directly caused by MV-induced H_2O_2 action, but H_2O_2 can be the result of MV-mediated cellular stress response. This is supported by the fact that similar levels of H_2O_2 accumulated by SNP treatment alone displayed no detrimental effects on leaf disks. Additionally, much higher H_2O_2 accumulation was detected in leaf disks simultaneously treated with MV and SNP. NO likely increases cellular H_2O_2, especially during the interaction with MV, modifying the cellular balance between O_2^- and H_2O_2 and preventing the induction of cell death. It has been shown that the cellular ratio between NOS and ROS is crucial for triggering PCD in soybean suspension-cultured cells (Delledonne et al. 2001).

In summary, NO was found to act as an antioxidant to regulate root development and PCD. NO also played a role in quenching ROS during oxidative stress. A large number of genes are regulated by NO at the transcriptional level in *Arabidopsis* (Parani et al. 2004; Polverari et al. 2003), which has prompted us to start an investigation into the functional role(s) of NO in regulating Chinese cabbage genes. In addition, it would also be interesting to determine the roles of antioxidant enzymes like SOD and catalase in the cellular homeostasis of RNS and ROS in Chinese cabbage.

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