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ORIGINAL RESEARCH

Mechanisms of Extracellular NO and Ca^{2+} Regulating the Growth of Wheat Seedling Roots

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Abstract Our previous studies suggested the cross talk of nitric oxide (NO) with Ca^{2+} in regulating stomatal movement. However, its mechanism of action is not well defined in plant roots. In this study, sodium nitroprusside (SNP, a NO donor) showed an inhibitory effect on the growth of wheat seedling roots in a dose-dependent manner, which was alleviated through reducing extracellular Ca^{2+} concentration. Analyzing the content of Ca^{2+} and K^+ in wheat seedling roots showed that SNP significantly promoted Ca^{2+} accumulation and inhibited K^+ accumulation at a higher concentration of extracellular Ca^{2+}, but SNP promoted K^+ accumulation in the absence of extracellular Ca^{2+}. To gain further insights into Ca^{2+} function in the NO-regulated growth of wheat seedling roots, we conducted the patch-clamped protoplasts of wheat seedling roots in a whole cell configuration. In the absence of extracellular Ca^{2+}, NO activated inward-rectifying K^+ channels, but had little effects on outward-rectifying K^+ channels. In the presence of 2 mmol L^{-1} $CaCl_2$ in the bath solution, NO significantly activated outward-rectifying K^+ channels, which was partially alleviated by $LaCl_3$ (a Ca^{2+} channel inhibitor). In contrast, 2 mmol L^{-1} $CaCl_2$ alone had little effect on inward or outward-rectifying K^+ channels. Thus, NO inhibits the growth of wheat seedling roots likely by promoting extracellular Ca^{2+} influx excessively. The increase in cytosolic Ca^{2+} appears to inhibit K^+ influx, promotes K^+ outflux across the plasma membrane, and finally reduces the content of K^+ in root cells.

Keywords Calcium · Nitric oxide · Plasma membrane K^+ channels · Wheat seedling

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Introduction

Nitric oxide (NO) is a highly diffusible gas and a ubiquitous bioactive molecule with well-characterized signaling roles in mammalian systems (Furchgott 1995). Recent evidence indicates that NO regulates a wide range of plant processes from development to environmental adaptation (Delledonne et al. 1998; Garcia-Mata et al. 2003; Gould et al. 2003), but its site of action in any signaling pathway remains unknown. In organ development of plant roots, NO can replace the role of auxin by activating its downstream MAPK system to mediate growth and development of lateral and adventitious root (Correa-aragunde et al. 2004; Pagnussat et al. 2002). In addition, NO increases the main root length in tomato and corn (Correa-aragunde et al. 2004; Pagnussat et al. 2004). However, the mechanism of NO regulating the growth of plant roots was still largely unknown.

Ca^{2+} is involved in abscisic acid- and hydrogen peroxide (H_2O_2)-induced stomatal closure as a versatile intracellular messenger (Mcainsh et al. 1990; Pei et al. 2000). Diverse biotic and abiotic stresses elicit a transient increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) (Knight et al. 1997; Zhao et al. 2008), and plants percept and decode these changes in $[Ca^{2+}]_{cyt}$, leading to specially physiological events (McClung 2006). Previous studies have shown that Ca^{2+} regulated the development of plant roots, such as the activity of plasma membrane Ca^{2+} channel, and the elevation of $[Ca^{2+}]_{cyt}$ is necessary for root growth and root

hair formation(Clementi 1998; Schiefelbein et al. 1992). Root absorption of Ca^{2+} is primarily through the root elongation zone and by the plasma membrane hyperpolarization-activated cation channel regulation (Kiegle et al. 2000; Very & Davies 2000). In *Arabidopsis thaliana*, increasing evidence has illustrated that $[Ca^{2+}]_{cyt}$ oscillations are synchronized to $[Ca^{2+}]_{ext}$ oscillations largely through the Ca^{2+}-sensing receptor CAS, and CAS regulates concentrations of inositol 1,4,5-trisphosphate, which evokes the release of Ca^{2+} from internal stores (Tang et al. 2007). This finding is distinct from McAinsh group's report that extracellular Ca^{2+} induced the elevation of $[Ca^{2+}]_{cyt}$ depending on the Ca^{2+} influx (Mcainsh et al. 1995). However, the mechanism of extracellular Ca^{2+} regulating root growth is not clear.

Previous researches have suggested that NO plays an important role in controlling Ca^{2+} channel activity and monitoring the balance of intracellular Ca^{2+} in animal cells (Besson-Barda et al. 2008; Clarkson and Hanson 1980). The cross talk between NO and Ca^{2+} forms an intricate network and participates in the regulation of a variety of physiological processes (Berridge et al. 2000; Besson-Barda et al. 2008). In the plant defense response, cGMP and cADPR are involved in the NO-mediated signaling pathway (Durner et al. 1998) as important factors that trigger the initiation of intracellular Ca^{2+} signaling pathways (Lee 2001). Our researches have revealed the cross talk of NO with Ca^{2+} in regulating stomatal movement (Zhang et al. 2009). However, it is unknown whether NO regulates the growth of root by regulating intracellular Ca^{2+} balance or not. Garcia-Mata et al. (2003) reported that NO regulated inward-rectifying K^+ channel of plasma membrane by the release of Ca^{2+} from intracellular Ca^{2+} stores in *Vicia* guard cells. Sokolovski and Blatt (2004) reported that NO efficiently inhibited outward-rectifying K^+ channel of plasma membrane, which is dependent on the extracellular Ca^{2+} influx. Recently, we found that NO effectively activated plasma membrane K^+ channels to promote K^+ absorption in the epidermal cells of wheat roots (Wen et al. 2008). Nevertheless, whether extracellular Ca^{2+} is involved in the regulations of NO on plasma membrane K^+ channels still remains poorly understood in plant root cells. To clarify the mechanism of NO and Ca^{2+} in the regulation of root growth, here, we investigated the regulation of extracellular NO and Ca^{2+} on root growth, plasma membrane K^+ channels, and the accumulation of cytosolic Ca^{2+} and K^+ in wheat seedling roots.

Materials and Methods

Plant Materials

Seeds of wheat (*Triticum aestivum* L.) for "yumai 49" were used in this study. For seed germination, all seeds were sterilized with 0.1% $HgCl_2$ and sown on 0.6% agar containing MS medium, then kept for 3 days at 4°C in the dark to break dormancy. The plates were then transferred to a culture room at 22°C and with a 16-h light/8-h dark photoperiod. For seedling growth, 3-day-old seedlings from the germination medium were transferred to sterile culture bottles containing 0.8% agar and 1/2 MS medium supplemented with various SNP or Ca^{2+} concentrations as indicated. For morphological examination, the culture bottles were incubated for 8 days at a day/night cycle of 12 h/12 h (0.20–0.30 mmol m^{-2} s^{-1}), and the temperature was kept at 22±2°C for day and 18±2°C for night, respectively.

Measurements of the Length and Number of Wheat Seedling Roots

The length and number of wheat seedling roots were determined as described by Wen et al. (2008) with slight modifications. Wheat seedling growth for 8 days was washed clean and naturally straightened to measure the length of the longest root of wheat seedling, and then the numbers of wheat seedling roots were recorded. Each value is the mean of 30 measurements ± standard error (n = 5).

Determination of K^+ and Ca^{2+} in Roots

K^+ and Ca^{2+} in seedling roots were determined as described previously (Zhao et al. 2007). The roots of wheat seedling were rinsed with deionized water three times and then dried at 80°C to a constant weight after filtration with Whatman paper. A total of 0.1 g dry powder samples was then extracted with 5 mL 4 mol L^{-1} HCl at 37°C overnight to release the free cations and centrifuged at 10,000×g for 10 min. The resulting supernatants of the extracts were diluted and K^+ and Ca^{2+} were determined with a Z-8000 atomic absorption/flame spectrophotometer.

Isolation of Root Cell Protoplasts and Whole Cell K^+ Current Recordings

Protoplasts were prepared from 8- to 10-day-old roots of "yumai49" (*T. aestivum*), as described previously (Findlay et al. 1994). Roots were briefly washed in deionized water before being removed from the plant. After removing the tips, the cortex was stripped from the stele by hand. The tissue was finely chopped, which was enzymatically digested for 2 h in a solution containing 0.8% cellulase (Yakult Honsha Tokyo Japan), 0.08% pectolyase (Sigma Chemical), 0.25% BSA, 0.5 mmol L^{-1} ascorbate, pH 6, and osmolality at 650 mOmol kg^{-1} adjusted with sorbitol, was then filtered using 50-mm nylon mesh, and centrifuged at 60×g for 8 min. The protoplasts could be maintained in ice-cold solution (10 mmol L^{-1} K-glutamate, 2 mmol L^{-1} $MgCl_2$, 1 mmol L^{-1}

KOH, 10 mmol L^{-1} Mes, 0.1 mmol L^{-1} $CaCl_2$, pH 6, and osmolality at 700 mOmol kg^{-1} adjusted with sorbitol) and stored on ice before patching experiments. In addition, we were able to distinguish between protoplasts from cortical cells and those from xylem parenchyma, as described before (Findlay et al. 1994). In any root, the number of cortical cells is very much greater than the number in the xylem parenchyma. We were more likely to have been using protoplasts from the cortex.

Whole cell K^+ current recordings were performed as described by Hamill et al. (1981), with some modifications. The protoplasts were placed in bath solutions containing (except where otherwise mentioned, such as 10 and 50 μmol L^{-1} SNP or 50 μmol L^{-1} SNP + 1 mmol L^{-1} $LaCl_3$ for treatments, respectively) 10 mmol L^{-1} K-glutamate, 2 mmol L^{-1} $MgCl_2$, 1 mmol L^{-1} KOH, 10 mmol L^{-1} Mes, and 0.1 mmol L^{-1} $CaCl_2$, pH 6. In addition, 0.1 mmol L^{-1} $CaCl_2$was abolished from the above bath solution for the absence of Ca^{2+}. In both cases (0.1 mmol L^{-1} Ca^{2+} or not), the osmolarity was adjusted to 700 mOsmol kg^{-1} with sorbitol. Pipettes were pulled with a vertical puller (model PC-10; Narishige) modified for two-stage pulls and fire-polished by a microforge (model MF-90; Narishige) before using. The pipette solution typically contained 100 mmol L^{-1} K-glutamate, 2 mmol L^{-1} $MgCl_2$, 4 mmol L^{-1} KOH, 1.1 mmol L^{-1} MgATP, 0.1 mmol L^{-1} $CaCl_2$, 10 mmol L^{-1} HEPES, pH 7.2, and osmolality at 720 mOsmol kg^{-1} with sorbitol. Data were acquired 15 min after the formation of the whole cell configuration. After the whole cell configuration was obtained, the membrane was clamped to −52 mV (holding potential). Whole cell currents were measured in response to 3-s voltage pulse from −190 to + 110 mV in 20-mV steps using an EPC-9 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany). Whole cell data were low-pass-filtered with a cutoff frequency of 2.9 kHz and analyzed with PULSEFIT 8.7, IGOR 3.0, and ORIGIN 7.0 software.

Results

Effects of Extracellular Ca^{2+} and NO on the Growth of Wheat Seedling roots

As shown in Fig. 1, SNP inhibited the growth of wheat seedling roots in a dose-dependent manner. SNP (50 μmol L^{-1}) inhibited the growth of wheat seedling roots by 75.29% and 100 μmol L^{-1} SNP inhibited by 85.87% with extracellular Ca^{2+} at a concentration of 10 mmol L^{-1}, which was alleviated through reducing the extracellular Ca^{2+} concentration, but extracellular Ca^{2+} alone had little effects on the growth of wheat seedling roots without SNP (Fig. 1a, b). However, the number of fibrous roots of wheat seedlings was less affected at different treatments (Fig. 1a, c). The results suggested that NO efficiently inhibited the growth of wheat seedling roots may be through promoting extracellular Ca^{2+} influx and increasing the accumulation of cytosolic Ca^{2+} in root cells.

Effects of Extracellular NO on Accumulation of Cytosolic Ca^{2+} of Wheat Seedling Roots

As shown in Fig. 2, NO elevated the Ca^{2+} content in wheat seedling roots. SNP (10 or 50 μmol L^{-1}) significantly promoted Ca^{2+} accumulation in wheat seedling roots, and the cytosolic Ca^{2+} was increased by 31.3% and 60.4%, respectively, with extracellular Ca^{2+} at a concentration of 10 mmol L^{-1}. Moreover, the increase in cytosolic Ca^{2+} depended on the extracellular Ca^{2+}. The results indicated that NO efficiently increased the accumulation of cytosolic Ca^{2+} probably via extracellular Ca^{2+} influx in root cells.

Effects of Extracellular Ca^{2+} and NO on K^+ Content of Wheat Seedling Roots

K^+ is the most abundant cation in plant cells and serves as an osmoticum, charge carrier, and enzyme cofactor (Chen et al. 2006). Since SNP inhibited the growth of wheat seedling roots in the presence of a higher concentration of extracellular Ca^{2+} (Fig. 1), we speculate that SNP inhibited K^+ accumulation in wheat seedling roots. As we speculate, 50 μmol L^{-1} SNP significantly inhibited K^+ accumulation in wheat seedling roots in the presence of 10 mmol L^{-1} extracellular Ca^{2+} (Fig. 3), and in the removal of Ca^{2+} from the growth medium, SNP promoted K^+ accumulation in wheat seedling roots. However, extracellular Ca^{2+} only had little effect on the K^+ content of wheat seedling roots at different concentrations. The results suggested that NO efficiently inhibited the absorption of K^+ by regulating Ca^{2+} absorption in wheat seedling roots.

Regulation of Extracellular Ca^{2+} or NO on Plasma Membrane K^+ Channels in Root Cells of Wheat Seedling

Since the cortical cells of roots are an important component of the route of uptake of nutrients from the soil to the plant and show uptake patterns similar to intact roots(Cram 1973), we choose the cortical cells for electrophysiological experiments to understand the mechanism of Ca^{2+}- or NO-regulated K^+ transport across membrane. The results showed that 10 or 50 μmol L^{-1} SNP efficiently inhibited the outward-rectifying K^+ channels and activated inward-rectifying K^+ channels in the absence of extracellular Ca^{2+} (Fig. 4). It is worth noting that 50 μmol L^{-1} SNP significantly activated outward-rectifying K^+ channels and inhibited the inward-rectifying K^+ channels when $CaCl_2$

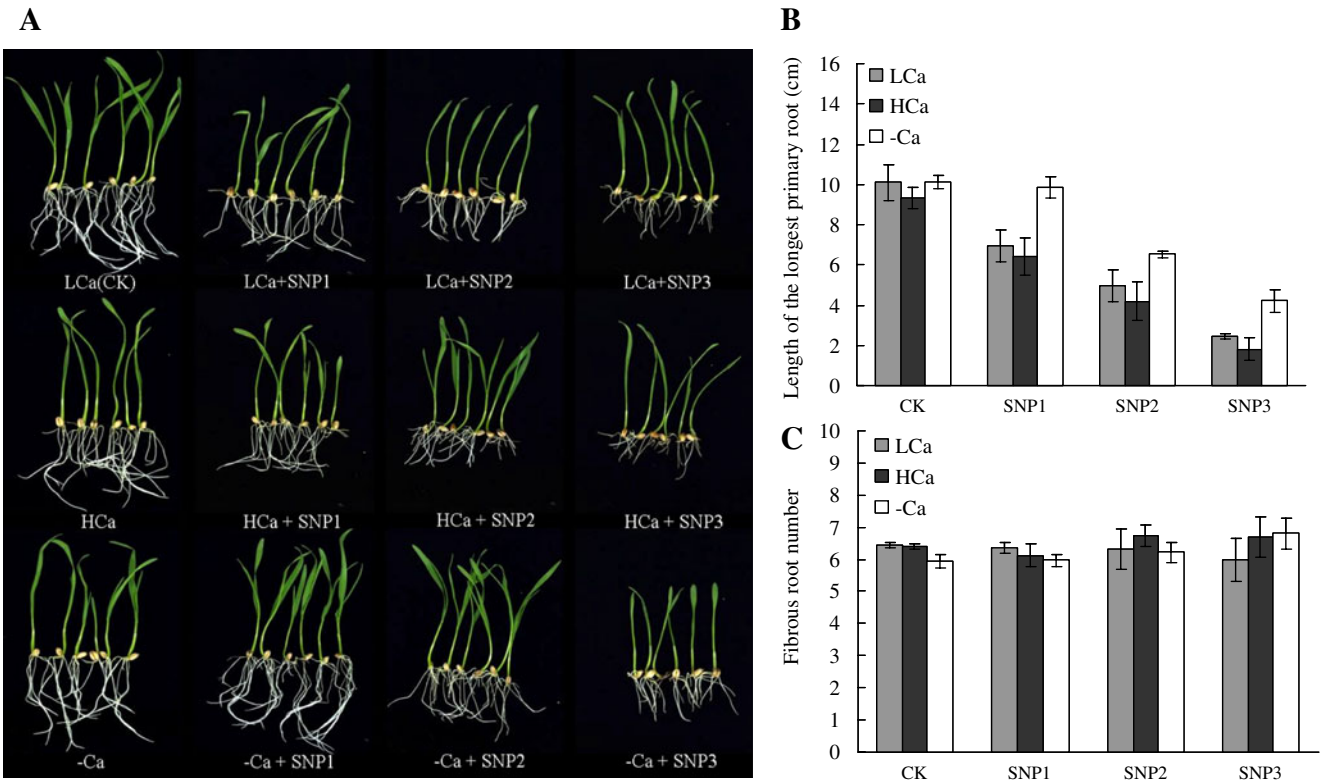

Fig. 1 Regulation of exogenous NO and Ca^{2+} on the growth of wheat seedling roots. **a** Effects of different treatments on the growth of wheat seedling roots. The concentrations of $CaCl_2$ used are 0 mmol L^{-1} for Ca, 2 mmol L^{-1} for LCa, and 10 mmol L^{-1} for HCa. The concentrations of SNP used are10 μmol L^{-1} for SNP1, 50 μmol L^{-1} for SNP2, and 100 μmol L^{-1} for SNP3. **b** Effects of exogenous NO and Ca^{2+} on the length of primary root. **c** Effects of exogenous NO and Ca^{2+} on length of fibrous number. Each value in **b** and **c** is the mean of measurements with standard error from six independent experiments

was added to the bath solution at concentration of 2 mmol L^{-1}.

Meanwhile, 50 μmol L^{-1} SNP significantly activated outward-rectifying K^+ channels and inhibited the inward-rectifying K^+ channels in the presence of 2 mmol L^{-1} $CaCl_2$, which was alleviated by La^{3+} at a concentration of 1 mmol L^{-1} (Fig. 5). In contrast, 2 mmol L^{-1} $CaCl_2$ alone had little effects on inward or outward-rectifying K^+ channels (Fig. 5). Therefore, we excluded the possibility of the effects of extracellular 2 mmol L^{-1} $CaCl_2$ itself on plasma membrane K^+ channels and confirmed that extracellular Ca^{2+} was involved in NO-regulated plasma membrane K^+ channels.

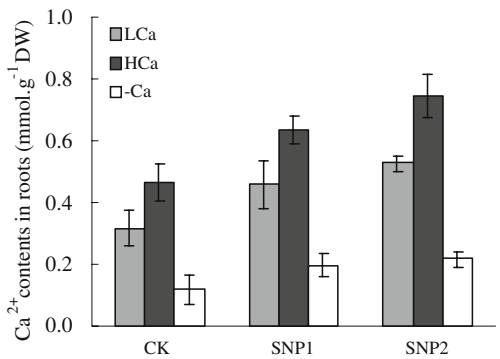

Fig. 2 Effects of exogenous NO on Ca^{2+} content in wheat seedling roots. Abbreviations are the same as in Fig. 1. Each *bar* represents the mean of measurements with standard error from six independent experiments

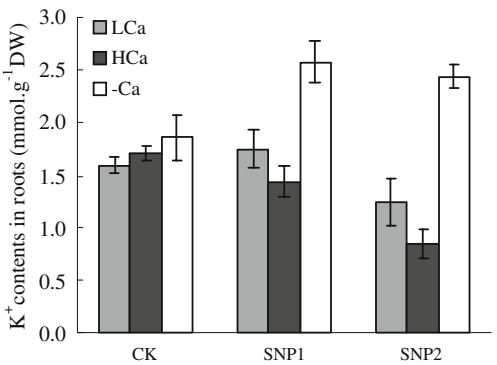

Fig. 3 Effects of exogenous NO and Ca^{2+} on K^+ content in wheat seedling roots. Abbreviations are the same as in Fig. 1. Each *bar* represents the mean of measurements with standard error from six independent experiments

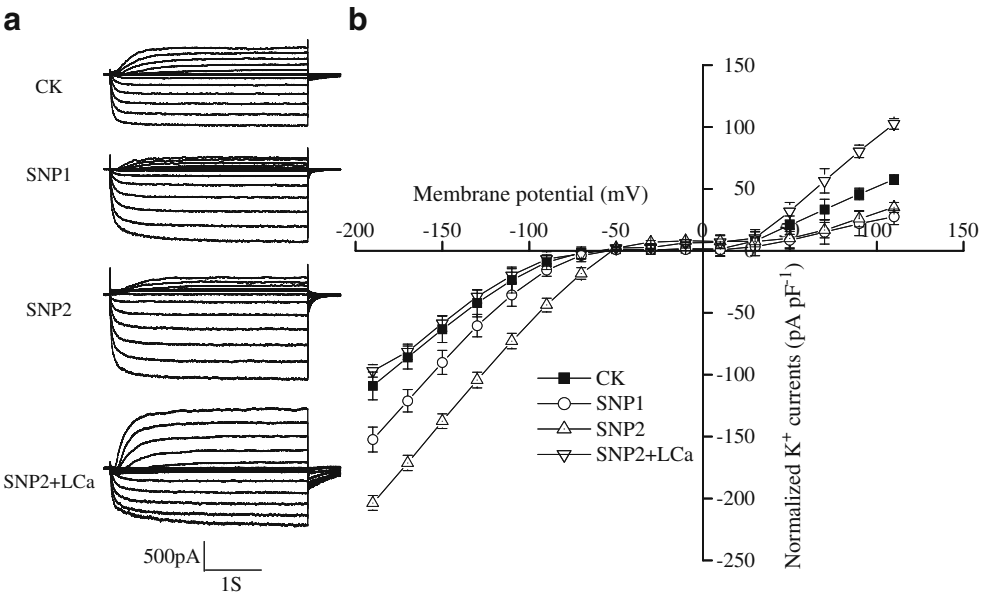

Fig. 4 Regulation of exogenous NO on inward-rectifying and outward-rectifying K^+ channels of the cortical cells in wheat seedling root. **a** Effects of different treatments on voltage-dependent inward- and outward-rectifying K^+ channels of the cortical cells in wheat seedling root. **b** Relationship between the whole cell K^+ current (pA) and membrane potential (mV). CK: control; SNP1: 10 μmol L^{-1} SNP; SNP2: 50 μmol L^{-1} SNP; SNP2+LCa: 50 μmol L^{-1} SNP + 2 mmol L^{-1} $CaCl_2$ treatments. Each value in **b** is the mean currents from six independent experiments, and the *error bar* denotes the standard error

Discussion

NO plays crucial roles in plant development, stress responses, and programmed cell death as a cellular mediator (Delledonne et al. 1998; Garcia-Mata et al. 2003; Gould et al. 2003). Increasing evidence indicates that NO replaces the role of auxin to mediate the development of lateral and adventitious root (Correa-aragunde et al. 2004; Pagnussat et al. 2002) and is also involved in root organogenesis (Pagnussat et al. 2004). In the present study, we demonstrated that NO showed an inhibitory effect on the growth of wheat seedling roots in the presence of a higher concentration of extracellular Ca^{2+} (Fig. 1). This finding is distinct from our previous reports that NO increases the length of primary roots and promotes root growth in wheat (Wen et al. 2008). A possible explanation might be that extracellular Ca^{2+} affects the regulating function of NO on the growth of wheat seedling roots. A key observation favoring the interpretation is that the inhibitory effects of NO on the growth of wheat seedling roots were alleviated by reducing extracellular Ca^{2+} concentration (Fig. 1). Furthermore, in the absence of extracellular Ca^{2+}, 10 μmol L^{-1} SNP slightly promotes the growth of wheat seedling roots. The results suggest that extracellular Ca^{2+} may be involved in the NO-mediated growth of wheat seedling roots and affects the regulating effect of NO on the growth of wheat seedling roots. This phenomenon was

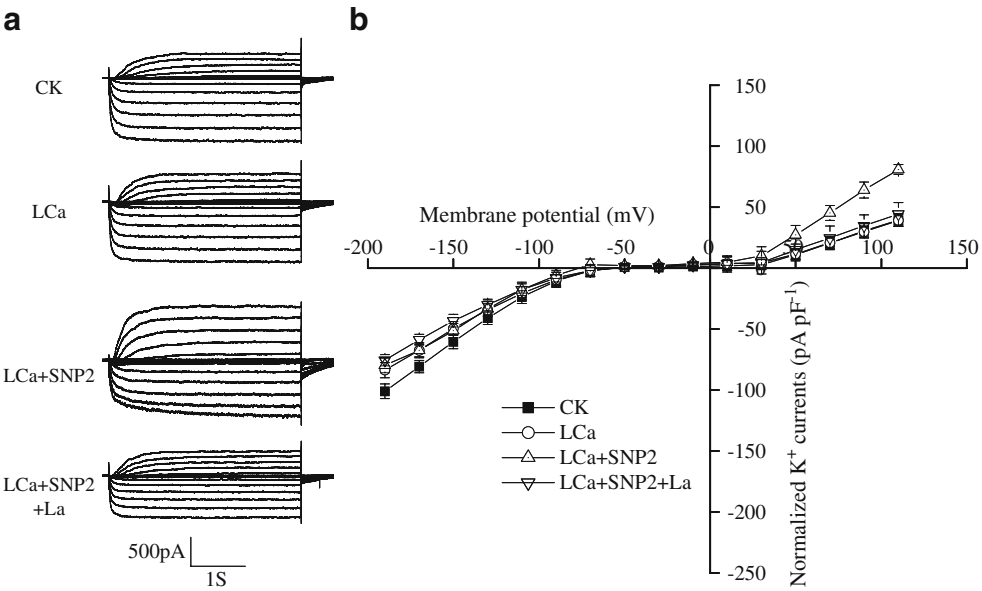

Fig. 5 Cross talk NO with Ca^{2+} in regulating K^+ channels of the cortical cells in wheat seedling root. The treatments of **a** and **b** are the same as in Fig. 4. CK: control; LCa: 2 mmol L^{-1} $CaCl_2$; LCa+SNP2: 2 mmol L^{-1} $CaCl_2$+100 μmol L^{-1} SNP; LCa+SNP2+La: 2 mmol L^{-1} $CaCl_2$+100 μmol L^{-1} SNP+1 mmol L^{-1} $LaCl_3$ treatments. Each value in **b** is the mean currents from six independent experiments, and the *error bar* denotes the standard error

further supported by those obtained that Ca^{2+} was involved in the development of plant roots, such as the activity of plasma membrane Ca^{2+} channel and the elevation of $[Ca^{2+}]_{cyt}$, which are necessary for root growth and root hair formation (Clementi 1998; Kiegle et al. 2000; Schiefelbein et al. 1992).

Ca^{2+} contributes to the physiological developmental events in plants as a ubiquitous second messenger in living cells (Besson-Barda et al. 2008; Clarkson and Hanson 1980; Lamotte et al. 2006; Mcainsh et al. 1990; Pei et al. 2000). Numerous studies indicate that diverse biotic and abiotic stresses elicit a transient increase in cytosolic free Ca^{2+} concentration $[Ca^{2+}]_{cyt}$ (Knight et al. 1997; Zhao et al. 2008), and plants percept and decode these changes in $[Ca^{2+}]_{cyt}$, leading to specially physiological events (McClung 2006). In animal cells, NO as a signal molecule plays an important role in controlling Ca^{2+} channel activity and monitoring the balance of intracellular Ca^{2+} (Clarkson & Hanson 1980). Our researches showed that NO promoted the influx of Ca^{2+} into the cytoplasm through Ca^{2+} channels in guard cells (Zhang et al. 2009). Moreover, the inhibitory effect of NO on the growth of wheat seedling roots is alleviated through reducing the extracellular Ca^{2+} concentration (Fig. 1). Therefore, we offer the hypothesis that NO efficiently inhibits the growth of wheat seedling roots may be through promoting extracellular Ca^{2+} influx and increasing the excess accumulation of cytosolic Ca^{2+} in root cells, To address this speculation, we investigated the effects of NO on the accumulation of cytosolic Ca^{2+} of wheat seedling roots. As we expected, NO obviously promotes Ca^{2+} accumulation in wheat roots (Fig. 2), which is accordant with those obtained that NO elevated the free cytosolic Ca^{2+} concentration by activating plasma membrane Ca^{2+} channels in *Nicotiana plumbaginifolia* cells (Lamotte et al. 2006). Although several lines of evidence have illustrated that elevated cytosolic Ca^{2+} can regulate reactive oxygen species production and pH changes during mechanosensing in *Arabidopsis* roots (Gabriele et al. 2009) and induce H_2O_2 accumulation under NaCl stress in guard cells (Zhao et al. 2008), the physiological function of elevated cytosolic Ca^{2+} in regulating the growth of plant roots is still largely unknown.

In guard cells, Schroeder's group has revealed that the elevated cytosolic Ca^{2+} is well characterized as a potential blocker of K^+ inward-rectifying channels and an activator of K^+ outward-rectifying channels (Schroeder and Hagiwara 1989), given that K^+ is the most abundant cation in plant cells and serves as an osmoticum, charge carrier, and enzyme cofactor to regulate the growth of plant (Chen et al. 2006). We speculate that Ca^{2+} is involved in NO-inhibited root growth likely by regulating K^+ uptake across the plasma membrane. Recently, some researches have suggested that plants absorb K^+ mainly through the plasma membrane K^+ channels (Findlay et al. 1994; Kochian et al. 1985) and the cortical cells of roots are an important component of the route of uptake of nutrients from the soil to the plant, and these cells' uptake patterns are similar to intact roots (Cram 1973). Here, we choose the cortical cells for electrophysiological experiments to understand the mechanism of K^+ transport across the membrane. As we speculate, in the presence of extracellular Ca^{2+}, NO significantly activated outward-rectifying K^+ channels and inhibited inward-rectifying K^+ channels to inhibit the K^+ accumulation in wheat seedling roots (Figs. 4 and 5), and in the absence of extracellular Ca^{2+}, NO efficiently activated inward-rectifying K^+ channels and inhibited the outward-rectifying K^+ channels to enhance the K^+ accumulation in wheat seedling roots (Figs. 3 and 4). However, 2 mmol L^{-1} $CaCl_2$ alone had little effect on inward or outward-rectifying K^+ channels (Fig. 5). Therefore, we excluded the possibility of that effect of extracellular 2 mmol L^{-1} $CaCl_2$ itself on the plasma membrane K^+ channels and confirmed that extracellular Ca^{2+} is involved in NO-modulating plasma membrane K^+ channels.

It was worth noting that the NO similar modulatory effect on outward-rectifying K^+ channels was acquired in the presence of extracellular La^{3+} (Fig. 5) and in the absence of extracellular Ca^{2+} (Fig. 4), which indicated that the increase in cytosolic Ca^{2+} was due to the extracellular Ca^{2+} influx rather than the release of Ca^{2+} from internal stores. Therefore, we infer that Ca^{2+} efficiently enhances the inhibition of root growth by NO, may be mainly through NO activating the plasma membrane Ca^{2+} channels. These findings are similar to our previous results that NO promoted influx of Ca^{2+} into the cytoplasm through Ca^{2+} channels to activate outward-rectifying K^+ channels in guard cells (Zhang et al. 2009).

Conclusion

In conclusion, NO inhibits the growth of wheat seedling roots likely by promoting extracellular Ca^{2+} influx excessively. The increase in cytosolic Ca^{2+} appears to inhibit K^+ influx and promotes K^+ efflux across the plasma membrane to reduce the K^+ accumulation in wheat seedling roots.

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