Short report

Comparison of effect of salt stress on the cell death in seminal root and lateral root of rye seedlings by the modified TUNEL method

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Abstract: Cell death in the lateral root tip of rye seedlings under salt stress conditions was analyzed quantitatively by the modified terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) method and the frequency of cell death was compared in the seminal root tip. There were no significant differences in total root length and the number of root tips among control, 10 mM and 100 mM NaCl treatments, although the root elongation and initiation was inhibited in the 250 mM NaCl treatment. The frequency of cell death was increased in 100 mM and 250 mM NaCl treatments compared with the control, significantly. There was no significant difference in the frequency of cell death between seminal root and lateral root in all stress treatments. Moreover, technical advantage of the modified TUNEL method was discussed by comparing with the classical TUNEL method.

Keywords: Cell death, Lateral root, Rye (*Secale cereale* L.), Salt stress, Seminal root, TUNEL method

Introduction

Root has roles of water and nutrients absorption and the condition of root is determined by the growth of plant. The severe stress conditions lead to death of cells in root. Therefore, it is important to estimate the degree of cell death in root for the understanding of plant growth under stress conditions.

In the early stage of cellular injury before apoptosis-like cell death, DNA is cleaved to 180-200 bp fragments (Katsuhara and Kawasaki, 1996). Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) is available for the in situ detection of DNA fragmentation. In this method, fluorescein-12-dUTP is incorporated into the 3'-OH DNA ends using the terminal deoxynucleotidyl transferase, recombinant, (rTdT) enzyme. The fluorescein-12-dUTP-labeled DNA, in which DNA fragmentation occurred, can be visualized directly by fluorescence microscopy. Katsuhara (1997) reported that DNA fragmentation in the seminal root of barley occurred after one hour of 500 mM NaCl treatment, and the TUNEL-labeled cells were increased with duration of the treatment. Ogawa et al. (2006) also investigated the cell death in seminal root tip of rye seedlings under some degree of salt stress conditions by the TUNEL method, quantitatively. Cell death was hardly observed from 0 mM (control) to 100 mM of NaCl treatments for one day. On the other hand, 85.9% cells in root tip was lead to cell death under the 250 mM of NaCl treatment. The degree of cell death was significantly higher than control (4.21%).

Root system changes its morphology to adjust itself to environmental stress, such as, water deficit and salt stress. It is called phenotypic plasticity. It is considered that the phenotypic plasticity is contributed to the plant growth through the maintenance of absorption of water and nutrients by the enlargement of root zone under the water deficit and salt stress conditions. The expression of phenotypic plasticity under stress conditions is highly affected by the growth of lateral roots because the root system consists largely of lateral roots (Bañoc et al., 2000). Yamauchi et al. (1996) reported that the change in lateral root development, i.e. the plasticity of the root system, exhibited under water deficit conditions may play an important role in drought tolerance.

Therefore, it is important to estimate the degree of

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cell death in lateral root for the understanding the effect of stress on root system development and plant growth. However, in previous studies, the change of cell death was investigated only at the seminal root tip, and not at the lateral root. In most of the previous studies, longitudinal resin sections were made and they were stained by TUNEL method to detect the cell death in root tip (Katsuhara, 1997; Katsuhara and Kawasaki, 1996; Ogawa et al., 2006). The advantage of this method is to enable us to observe the cell death using an organ part or tissue at a cellular level on the section. However, it needs a hard work to prepare longitudinal resin sections at the tip of lateral root. Unless a longitudinal section containing the center of the root axis is observed, proportions of each root tissue included in a section depend on where in the root the section is obtained. Therefore, a precise assessment should be difficult particularly when frequency of cell death depends on the tissues. To overcome this shortcoming of the classical method, we have developed the modified TUNEL method, in which tissues were dissociated enzymatically so that the cell death can be assessed regardless of the tissues.

The objective of this study is to investigate the cell death quantitatively in the lateral root tip under salt stress by the modified TUNEL method and to compare the effect of salt stress to the cell death in the seminal root tip. Moreover, the effectiveness of the modified TUNEL method was tested by comparing with the classical TUNEL method.

Materials and Methods

Seeds of rye (*Secale cereale* L.) were germinated in the dark at 28ºC in petri dishes for 3 days. During this time, the seminal root elongated approximately 10 mm in length. The seedlings were transplanted onto plastic nets floating in a one-liter beaker filled with nutrient solution. The nutrient solution contained 3.0×10^{-3} M KNO_3, 2.0×10^{-3} M $Ca(NO_3)_2$, 5.0×10^{-4} M $NH_4H_2PO_4$, 1.0×10^{-3} M $MgSO_4$, 2.6×10^{-5} M Fe-EDTA, 4.6×10^{-6} M $MnCl_2$, 2.4×10^{-5} M H_3BO_3, 3.8×10^{-7} M $ZnSO_4$, 1.6×10^{-7} M $CuSO_4$ and 1.5×10^{-8} M $(NH_4)_6Mo_7O_{24}$. The nets were covered with aluminum foil with pinholes to allow roots to grow in the dark. The nutrient solution was aerated sufficiently. The plants were grown under a 12-hour photoperiod in a growth chamber (MLR-350H, SANYO, Osaka, Japan). The photon flux density of photosynthetically activity radiation (PAR, 400-700 nm) was 320 μmol m^{-2} s^{-1}. The chamber was maintained at 18ºC with 80% relative humidity during the day and night. After 6-day of the transplanting, NaCl were added to the nutrient solution for the salt stress treatments and the NaCl concentration were adjusted to 0, 10, 50, 100 and 250 mM, respectively.

After 24 hours of NaCl treatments, 2 mm length of root tip regions of seminal roots or lateral roots were sampled and fixed in the 4% (w/v) paraformaldehyde for over night. These sampled seminal and lateral roots were already emerged and elongated during the stress treatments. The fixed root segments were washed with deionized water for 30 minutes, transferred into a plastic tube of 2 ml volume and treated with disassociation solution (2% (w/v) cellulase (MP Biomedicals, Inc, Illkirch, France) and 2% (w/v) pectinase (Fluka Biochemika, Buchs, Switzerland) were dissolved in aqueous solution containing 6.8×10^{-3} M $CaCl_2$, 0.4% (w/v) polyvinylpyrrolidone K30, 0.6 M mannitol and 1.1×10^{-2} M $K_2H_2P0_4$, pH 5.8). The root segments were warmed in the water bath at 37˚C for 60 minutes and then disassociated root segments were washed with deionized water for 40 minutes in petri dishes. The disassociated root segments were placed on slide glass, a few drops of 4% (w/v) solution of paraformaldehyde was added to them, a coverslip was placed on them and the coverslip was tapped several times from the upper surface to flatten the root segment. Then the coverslip was peeled off carefully and the slide glass was dried at 37 ºC for over night. The dried slide glass was used for the detection of cell death.

The detection and the observation of cell death by the TUNEL labeling and the quantitation of the cell death frequency were followed by the methods as described previously (Ogawa et al., 2006).

For the measurement of total root length and the number of root tips, sampled roots were immediately fixed and stained with 0.1 % (w/v) Coomassie Brilliant Blue G250 dissolved in FAA (5% formaldehyde: 50% ethanol: 5% acetic acid [v/v]), and kept for two days. The images of the root systems were captured by an image scanner (GT-9800F Epson Co. Ltd., Tokyo, Japan), and total root length and the number of root tips were measured by WinRHIZO software (Regent Instrument Inc., Quebec, Canada).

Results and Discussion

In this study, we investigated the cell death quantitatively in the rye seedlings under salt stress conditions in the tip of lateral root which construct the large part of root system and compared the effect of salt stress on the cell death in the seminal root tip.

Figure 1 showed the total root length and the total number of root under salt stress. There were no significant differences in total root length among control, 10 mM and 100 mM NaCl treatments (Fig. 1A). The total root length increased 42.5 cm, 45.0 cm and 57.4 cm during 24 hours compared with the total root length before the stress treatments, when the

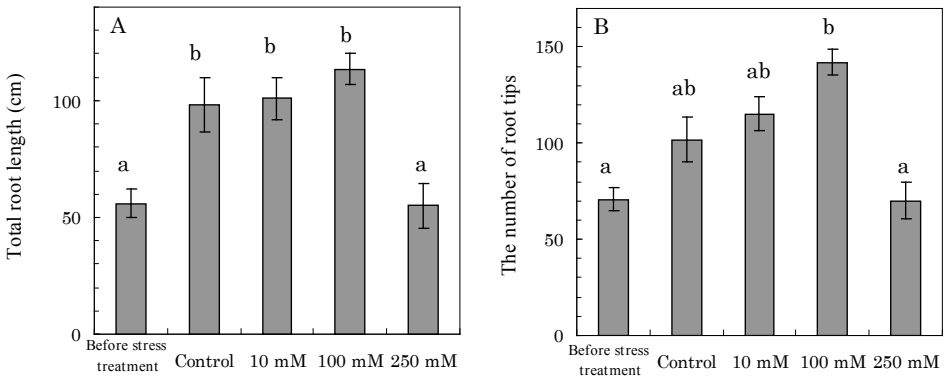

Fig. 1. Effect of salt stress on the total root length (A) and the number of root tips (B). Each value shows the mean ± standard error (n= 6-12). Means followed by the common letters were not significantly different according to the multiple test of Tukey (P<0.05).

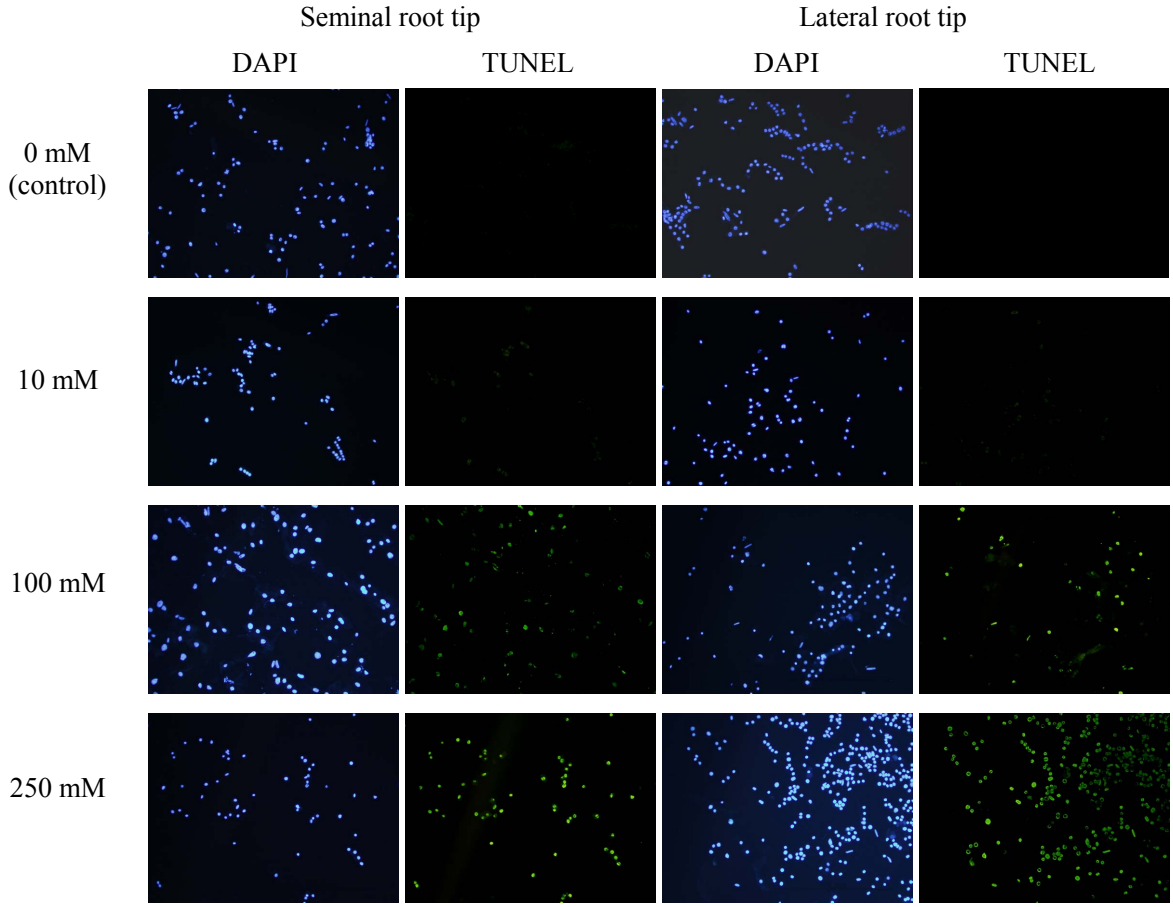

Fig. 2. Micrographs of TUNEL-labeled cells and cells stained with DAPI at seminal root tips and lateral root tips treated with 0 mM (control), 10 mM, 100 mM and 250 mM NaCl.

roots were treated with 0 mM, 10 mM and 100 mM NaCl, respectively. There was a tendency that the total length was increased with the increase of stress strength among these treatments. On the other hand, at the 250 mM NaCl treatment, root elongation was inhibited significantly compared with the other treatments, and there was no elongation during 24 hours after the stress treatment. The total number of

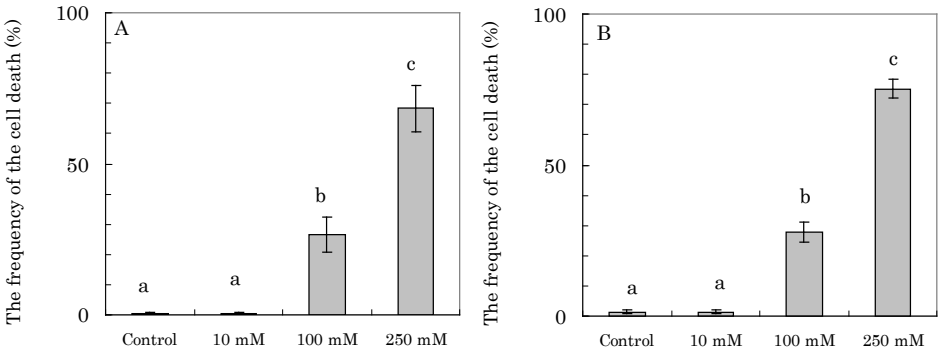

Fig. 3. Effect of salt stress on the frequency of cell death estimated by TUNEL labeling in seminal root (A) and lateral root (B). Each value shows the mean ± standard error (n=10). Means followed by the common letters were not significantly different according to the multiple test of Tukey (P<0.05). There were no significant differences in the frequency of cell death between seminal root and lateral root in the NaCl treatments at the same concentration.

roots was increased with the increase of stress strength from the control upto 100 mM treatment, although there were no significant differences (Fig 1B). In 250 mM treatment, the total number of roots was not increased during 24 hours and was lower than that of the 100 mM NaCl treatment, significantly.

The micrographs of the cells labeled with TUNEL at seminal root tips and lateral root tips under salt stress were shown (Fig. 2). TUNEL-labeled cells were hardly detected both in seminal root and lateral root treated with 10 mM NaCl for 24 hours as in the control. In the 100 mM NaCl treatment, some cells were labeled by TUNEL in seminal root and lateral root tips. In the 250 mM NaCl treatment, many TUNEL-labeled cells were detected both in seminal and lateral root tips, and DNA fragmentation was seen in most of the cells observed.

The frequency of cell death under salt stress was calculated from the ratio of the number of TUNEL-labeled cells to the number of all cells stained with DAPI (Fig. 2) in root tips of seminal root and lateral root (Fig. 3). In the seminal root tip, the frequency of cell death was low in the 10 mM NaCl treatment (0.48%), and there was no significant difference compared with the control (0.44%) (Fig. 3A). The frequency of cell death was increased in the 100 mM (26.6%) and 250 mM (68.3%) NaCl treatments, significantly. In the lateral root tip, the change of the frequency of cell death was similar to that in the seminal root (Fig. 3B). The frequency of cell death in lateral root of control, 10 mM, 100 mM and 250 mM NaCl treatments were 1.35%, 1.43%, 27.9% and 75.2%, respectively. In all of the treatments, there were no significant differences in the frequency of cell death between seminal root and lateral root in the NaCl treatments at the same concentration.

In the classical method, sections were used to detect and estimate the degree of the cell death in root tip (Katsuhara, 1997; Katsuhara and Kawasaki, 1996; Ogawa et al., 2006). In the present study, we estimated the cell death in the root tip by the modified TUNEL method, exhaustively. In our previous study (Ogawa et al., 2006), the cell death in seminal root tip of rye seedlings under some degree of salt stress conditions were also investigated using the classical TUNEL method. The frequency of cell death in the 0 mM, 10 mM NaCl treatments detected by the classical TUNEL method were similar to those detected by the modified TUNEL method in the present study. However, the frequency of cell death in the 100 mM and 250 mM NaCl treatments detected by the classical method was 1.2% and 85.9%, respectively. These values were different from those detected by the modified method in the present study (26.6% in the 100 mM NaCl treatment and 68.3% in the 250 mM NaCl treatment). There is a possibility to misestimate the frequency of cell death detected by the classical method using the longitudinal section, especially when the section was obtained in outer part of root axis, because of the reason mentioned in the Introduction.

In the 250 mM NaCl treatment, the inhibition of elongation and initiation of root (Fig. 1) were occurred in connection with cell death in seminal root and lateral root (Fig. 3). On the other hand, in the 100 mM NaCl treatment, there were no differences in the elongation and initiation of root compared with the control (Fig 1), although the approximately 30% of the total cells in the tips of seminal root and lateral root were died (Fig. 3). The sampled seminal and lateral roots, in the present study, were already

emerged and elongated during the stress treatments. It was reported that the cell division was promoted by 100 mM NaCl treatment in the seminal root tip of rye seedlings (Ogawa et al., 2006). These results suggested that the cell division and the root initiation were promoted in the roots emerged during 100 mM treatment, and, therefore, the growth of root system was compensated. Further studies are required to clarify the difference of responsibility to the stress due to a difference in the point of the root emergence.

In this study, we investigated the root growth and the cell death quantitatively in the lateral root tip under salt stress and compared the effect of salt stress on the cell death in the seminal root tip using the modified TUNEL method. Further studies are required to clarify the effect of cell death on the root system formation under the environmental stress conditions.

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