Intervarietal Differences in the Occurrence of Internal Browning and the Role of Ascorbic Acid in *Raphanus* Roots

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The present study was carried out to compare the differences in peroxide-scavenging metabolism between radish cultivars which are susceptible to internal browning (IB) colorization upon increased temperature and cultivars which are resistant to browning. The induction of IB in the susceptible cultivars was progressively intensified with high temperature treatment during the later growth period, resulting in significant increases of glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6PGD), L-phenylalanine ammonia-lyase (PAL), and polyphenol oxidase (PPO) at the central stele. Conversely, in the IB-resistant ones, neither the accumulation of IB nor induction of these enzymes occurred regardless of the treatment during this period. No correlation was found between the isothiocyanate (ITC) content and the development of IB. The ascorbic acid (AsA) content, however, was closely correlated with the development of IB. These results strongly suggest that intervarietal differences with respect to susceptibility to IB formation are closely correlated with differences in the polyphenol biosynthesis ability under high temperature conditions and largely dependent on AsA metabolism in the root.

Key Words: ascorbic acid, internal browning, intervarietal difference, polyphenol biosynthesis.

Introduction

Internal browning in *Raphanus* roots, consisting of a brown coloration at the central region of the root, is a physiological disorder caused by heat stress during the root maturation period (Fukuoka and Kano, 1990; Kawashiro, 1990; Kawashiro and Takeda, 1988). According to Kawai et al. (1992), increased severity of IB formation was closely correlated with the accumulation of phenolic compounds in the internal region. It was reported that thermal conditions above the optimum range often resulted in a pronounced increase of enzymes associated with polyphenol biosynthesis, stimulating the induction of IB formation in *Raphanus* roots (Fukuoka and Enomoto, 1996). As for this phenomenon, it is suggested that a decline in the H$_2$O$_2$-decomposing capacity of the ascorbate-glutathione cycle is responsible for activation of the H$_2$O$_2$-detoxifying pathway derived from polyphenol biosynthesis (Fukuoka and Enomoto, 2001). Accordingly, the H$_2$O$_2$ level in roots of IB-susceptible cultivars exposed to heat stress is increased by disrupting the AsA regeneration system in the ascorbate-glutathione cycle. And, peroxide-scavenging systems coupled with polyphenol biosynthesis are induced in order to detoxify active oxygen species instead of the ascorbate-glutathione cycle. They also indicated that IB-resistant cultivars retain their H$_2$O$_2$-decomposing capacity via the ascorbate-glutathione cycle even when roots are subjected to heat stress during the root maturation period, and then IB symptoms seldom occur (Fukuoka and Enomoto, 2001). Furthermore, Kawashiro (1990) and Kawashiro and Takeda (1988) reported that intervarietal differences exist with respect to susceptibility to IB formation. These findings strongly suggest that the different reactions for IB among cultivars largely depend on the AsA level in the root. The present investigation was carried out to clarify the relationship between the frequency of IB occurrence among cultivars and its physiological function, and demonstrated that intervarietal differences were largely dependent on the oxygen scavenging ability coupled with the ascorbate-glutathione cycle.

Materials and Methods

1. Plant materials

Seeds of *Raphanus sativus* L. ‘Syukhou’, ‘Akimine’,

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‘Akiou’, ‘Ajitenka’, and ‘Fukutenka’ were sown on 5 August 1997 in the field of the experimental farm of Ishikawa Sand Dune Agricultural Experimental Station, Japan. Sixty g m⁻² of dolomite were applied at the end of June and 25 g m⁻² of N, P₂O₅, and K₂O and 60 g m⁻² of micro elemental manure containing 0.4% Mn, 0.3% B, 1.2% Fe, and 0.03% Cu were applied in early August. Each cultivar was sown in 2 experimental plots, consisting of a control plot and a high temperature plot (HTP). For the HTP, a tunnel 40 cm high and 70 cm wide was laid above the row and this was covered with a 0.05 mm thick polyolefin film from 66 to 70 days after sowing. The control plot was not given any treatment. The daily maximum air temperatures in the HTP exceeded 35°C for the first 4 days of treatment, and were approximately 15°C higher than that of the control during the same period (Fig. 1). Likewise, the daily maximum soil temperatures in the HTP were always above 25°C during this period and were 12–14°C higher than those of the control. After high temperature treatment, 15 radishes were sampled randomly, and then 10 uniform roots from these were selected. To measure IB, roots were cut lengthwise at the central axis and the degree of browning was classified by visual inspection, as shown previously (Fukuoka and Enomoto, 2001).

2. Enzyme assays

To determine the G6PD and 6PGD activities, the central region of the pith was hollowed out and about a 10 g lump was used. Each sample was suspended with 3 volumes of ice-cold 20 mM Tris-HCl buffer (pH 7.5) containing 4 mM EDTA and 15 mM 2-mercaptoethanol, and homogenized with sea sand in a pestle. The homogenate was centrifuged at 10000×g for 30 min at 4°C. The G6PD and 6PGD activities were assayed in a reaction mixture (2.5 ml) containing 10 mM Tris-HCl buffer (pH 7.5), 10 mM MgCl₂, 0.5 mM NADP⁺, 2.5 mM glucose-6-phosphate, and crude enzyme solution by measuring the increase in absorbance of NADPH at 340 nm (Fukuoka and Enomoto, 1996). The PAL activity was measured as described by Cano et al. (1990). To determine the PAL activity, 0.1 g of polyvinylpolypyrrolidone was added to 1 g of frozen sample and homogenized in 5 mL of 100 mM borate buffer (pH 8.8). The homogenate was centrifuged at 10000×g for 30 min at 4°C. The reaction mixture consisted of 1 mL of the crude extract and 1 mL of 50 mM borate buffer (pH 8.8) containing L-phenylalanine (20 mM), and was incubated at 30°C for 30 min. The PAL activity was assayed by measuring the increase in absorbance of trans-cinnamic acid at 270 nm.

The PPO activity was assayed of the method by Koukol and Conn (1961). One gram of the lump added to 0.1 g of polyvinylpolypyrrolidone was homogenized in 5 mL of 50 mM phosphate buffer (pH 7.0). The homogenate was centrifuged as described above, and the supernatant was used as a crude extract. The PPO activity was assayed in a reaction mixture (2.1 mL) containing 50 mM Hepes buffer (pH 7.5), 1% catechol solution, and crude enzyme solution by measuring the increase in absorbance at 420 nm at 30°C.

3. Measurement of the ITC content

The ITC content was determined according to the method of Ezaki and Onozaki (1980). Two grams of the lump was homogenized in 10 mL distilled water. The homogenate was filtered through two layers of cotton gauze. The filtrate was put into a 20 mL bottle with a rubber cap, and was kept at 30°C for 30 min. To convert produced ITC into thiourea derivatives, an 8 mL mixture of ethanol and aqueous ammonia was added to 2 mL of extract, followed by maintaining it at 30°C for 60 min.

Fig. 1. Changes in air and soil temperatures during the high temperature treatment. A and B indicate the air and soil temperature, respectively. Upper and lower indicate the HTP and control, respectively.
The mixture was neutralized with 0.5 mL of 50% acetic acid. A mixture of 1 mL solution and 4 mL modified Grote reagent was incubated at 37°C for 45 min, and the optical density was determined at 600 nm.

4. Measurement of the AsA content

Assay of the AsA level was performed using HPLC. Ten grams of the lump was homogenized in 60 mL of 2% metaphosphoric acid. The homogenate was centrifuged at 10000×g for 15 min at 4°C. The supernatant was filled up to 100 mL with 2% metaphosphoric acid. After the sample was filtered through a 0.45 µm membrane filter, a 10 µL aliquot was injected onto a Shim-Pack column (SCR-101N, Shimazu, Japan) attached to a LC-10AD pump (Shimazu). The column, maintained at 40°C, was eluted with 50 mM NaOH at a flow rate of 1.0 mL·min⁻¹. After chromatographic separation, AsA was reacted with 50 mM NaBH₄ at a flow rate of 0.5 mL·min⁻¹ by post-label method. Ascorbic acid was monitored at 300 nm with a SPAD-10A spectrophotometric detector (Shimazu) attached to a chart recorder (C-R6A, Shimazu).

Results and Discussion

Several cultivars of radishes have been investigated concerning the frequency of IB occurrence as well as physiological functions concerned with it. In our experimental results, Japanese radishes could be divided from the level of IB disorder into two types. One is a type that develops IB at a high frequency, such as cultivars ‘Ajitenka’ or ‘Fukutenka’ (Fig. 2). The other, resistant to IB, was represented by ‘Syukuhou’, ‘Akimine’, or ‘Akiou’. The interesting and significant facts to be noted were that the former roots exhibiting IB were found to be more numerous when the temperature remained high during the later growth period, but in the latter, the thermal condition had no effect and IB rarely formed even under high temperature conditions during this period. According to Fukuoka and Enomoto (1996), high temperatures during the later growth period often resulted in a pronounced increase of enzymes associated with polyphenol biosynthesis, stimulating the induction of IB formation in Raphanus root. Furthermore, they (2001) pointed out that different cultivars of radishes show very different responsiveness to high soil temperature in relation to polyphenol biosynthesis, and that the differences in enzyme activities associated with this process are closely correlated with the susceptibility to IB occurrence. In this experiment, profiles of the changes in activities of these enzymes exhibited an identical pattern. As shown in Figure 3, there was a substantial induction of enzymes in the IB-susceptible cultivars. Accordingly, the activities of G6PD and 6PGD in the susceptible cultivars were about 2 times higher than those of the resistant ones. There were no significant differences in PAL activity between the susceptible cultivar ‘Ajitenka’ and resistant ones. However, typical PAL induction occurred in ‘Fukutenka’, which is most likely to develop IB symptoms. Results of PPO activity were similar to those for the G6PD and 6PDG activities. These results strongly suggest that intervarietal differences of susceptibility to IB formation under high temperature treatment are closely correlated with polyphenol biosynthesis activity.

Experimental results revealed that the AsA content proved to be a good indicator of IB occurrence in

![Fig. 2](image-url) Intervarietal differences in the occurrence of internal browning among several cultivars. Open and solid columns indicate the control and HTP, respectively.

![Fig. 3](image-url) Intervarietal differences in G6PD, 6PGD, PAL, and PPO activities after high temperature treatment. A, B, C, D, and E indicate ‘Syukuhou’, ‘Akimine’, ‘Akiou’, ‘Ajitenka’, and ‘Fukutenka’, respectively. Means followed by the same letter are not significantly different at the 5% level by Tukey’s multiple-range test.
Raphanus root. A larger amount of AsA was detected in the resistant cultivars in comparison with the level in the susceptible ones showing marked symptoms of IB (Fig. 4). The IB-susceptible cultivars contained AsA below 12 mg%, while the AsA level of resistant ones ranged from 17 to 25 mg%. Fukuoka and Enomoto (2001) suggested that the \( \text{H}_2\text{O}_2 \) level in roots of IB-susceptible cultivars exposed to heat stress is increased by the disruption of the AsA regeneration system in the ascorbate-glutathione cycle, and that peroxide-scavenging systems coupled with polyphenol biosynthesis are induced in order to detoxify active oxygen species instead of the ascorbate-glutathione cycle, bringing about the accumulation of brown substances in the pith region. The ascorbate-glutathione cycle is known to involve successive oxidations and re-reductions of ascorbate, glutathione, and NADPH by enzymes such as ascorbate peroxidase, dehydroascorbate reductase, and glutathione reductase. Here, the rapid regeneration of ascorbate by the cycle is an absolute necessity because ascorbate irreversibly converts to other metabolites like oxalic acid and is rapidly lost (Foyer et al., 1991). Johnson and Schaal (1957) observed the accumulation of phenolic material and AsA in white potato tuber tissue upon injury and suggested their possible role in resistance to injury. Many studies on the action of PPO in several plants showed that AsA performed the role of a reducing agent for \( \text{o-quinones} \) produced during the oxidation of substrates (Baruah and Swain, 1990; Fujita and Tono, 1988; Oba et al., 1994; Yamauti and Ogata, 1979). It seems, therefore, that if IB-susceptible cultivars are exposed to heat stress during the root maturation period, the roots synthesize only lower levels of peroxide-scavenging components coupled with the ascorbate-glutathione cycle owing to the inhibited regeneration system of AsA. This decline in the \( \text{H}_2\text{O}_2 \)-decomposing capacity via the ascorbate-glutathione cycle activates other scavenging systems in conjunction with polyphenol biosynthesis, and then brown substances accumulate in the pith region. Conversely, the IB-resistant cultivars retain their \( \text{H}_2\text{O}_2 \)-decomposing capacity via the ascorbate-glutathione cycle even when roots are subjected to heat stress during the root maturation period, and then IB symptoms seldom occur.

In this experiment, there was no correlation between intervarietal differences of IB occurrence and ITC products in roots (Fig. 5). Higher contents of ITC were detected in the IB-susceptible cultivars ‘Fukutenka’ or ‘Ajitenka’ as compared with the resistant ones, ‘Akiou’ or ‘Akimine’. But in ‘Syukuhou’, the ITC content was high in spite of resistance to IB formation. This finding strongly supported our previous assumption that the protective effect of ITC on IB occurrence was not nearly so marked (Fukuoka and Enomoto, 2001).

In conclusion, heat stress induced IB in the central stele of radish root. The degree of IB is closely correlated with the fluctuation in enzyme activities of polyphenol biosynthesis and the ascorbate-glutathione cycle. However, there are intervarietal differences in the occurrence of IB. These results strongly suggest that intervarietal differences with respect to susceptibility to IB formation induced by high temperature conditions are largely dependent on the AsA regeneration ability in the ascorbate-glutathione cycle of the root, and that accumulation of IB is an adaptive metabolic response that allows plants to survive the toxicity of active species of oxygen.

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