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[1]Department of Agricultural Chemistry, Institute of Environmentally-Friendly Agriculture (IEFA), College of Agriculture and Life Sciences, Chonnam National University, Gwangju, Korea
[2]Department of Horticulture, College of Agriculture and Life Sciences, Chonnam National University, Gwangju, Korea
[3]Asian Pear Research Institute, Chonnam National University, Gwangju, Korea

Changes in activity and isozyme patterns of peroxidase and chitinase in kiwifruit pollen

Yong-Su Song[1,#], Sang-Hyun Lee[2,3,#], Jung-An Jo[3], Seung-Hee Choi[1], Dong-Jun Seo[1], Yong-Kyu Lee[3], Ung Yang[3], Woo-Jin Jung[1,*]

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Summary

In this study, changes in activity and isozyme patterns of peroxidase (POD) and chitinase in kiwifruit (*Actinidia chinensis*) pollen were investigated under different storage conditions. Although residual activity was detected in heat-treated pollen, changes in POD activity were observed due to difference in storage conditions as revealed by preliminary studies in which pollen germination varied with different storage conditions. POD activity of kiwifruit pollen increased as proportions of viable pollen increased, indicating a positive correlation (R^2=0.993) between pollen viability and POD activity. There was a detectable difference in the relative activity of POD enzyme between heat-treated and viable pollen. Decoloration of Congo Red was observed in germination medium which fresh pollen was cultured. The activity of individual chitinase isozymes present in kiwifruit pollen differed depending on storage conditions, which had a direct impact on pollen vigor. Although direct evidence showing that chitinase isozymes are implicated in pollen vigor is still uncertain, distinction of isozymes may facilitate more precise identification of viable pollen which possesses germination potential from non-viable pollen. Taken together, these results suggest that monitoring the activity of POD and chitinase can be an attractive alternative to evaluate pollen vigor in kiwifruit.

Keywords: *Actinidia chinensis*, Chitinase isozymes, Kiwifruit pollen, Monitoring, POD activity, Viability

Introduction

Kiwifruit (*Actinidia chinensis* Planch) is a dioecious species and mainly wind-pollinated, but artificial pollination of kiwifruit is becoming a desirable alternative to ensure a satisfactory crop yield. It is necessary to determine the viability of stored pollen before its use in artificial pollination, since pollen viability that affected pollination efficiency may be reduced during storage period. Thus, developing a robust method that can be used routinely for verifying pollen vigor is important to assure successful pollination.

The longevity of pollens varies greatly with different plant species and is known to be affected by storage conditions such as temperature and humidity (Dafni and Firmage, 2000). To find optimal conditions for long-term preservation of pollen used in artificial pollination, effects of pollen storage under different temperature conditions on pollen viability have been studied in several plant species including caladium (Deng and Harbaugh, 2004), phalaenopsis (Yuan et al., 2018), hazel (Novara et al., 2017), date palm (Mesnoua et al., 2018), and kiwifruit (Borghezan et al., 2011).

The presence of peroxidases has been reported in the pollen wall of several plant species including lily (Dashek et al., 1979), poplar (Ashford and Knox, 1980), and kiwifruit (Speranza et al., 2012). Changes in peroxidase activity and isozyme pattern during pollen maturation and germination are well understood (Malik and Gupta, 1976; Parui et al., 1998; Dai et al., 2007), although pollen has relatively low peroxidase activity as well as low isozyme polymorphism compared to other plant tissues (Malik and Gupta, 1976; Bredemeijer, 1984). The majority of prior researches on peroxidase activities have focused to how they contribute to physiological processes associated with pistil-pollen interaction (Bredemeijer, 1984). Interestingly, peroxidase activity in cross-pollinated tobacco styles increases steadily during pollen tube growth (Bredemeijer, 1974) while peroxidase activity in pistils pollinated with non-viable pollen was shown to increases irrespective of the penetration of pollen tube in *Pyrostegia venusta* (Chaudhary et al., 2010). This indicates their role in defense response (Pandey et al., 2017) rather than being implicated to stigma receptivity (Dafni and Maués, 1998). Indeed, among proteins induced during plant defense, class III plant peroxidases are known to be implicated in cell wall rigidification through cross-linking of several compounds such as lignin, suberin, extensins and polysaccharide-linked ferulates (Pandey et al., 2017).

Most plant peroxidases belong to class III heme-containing peroxidases (EC 1.11.1.7) which catalyze reactions that involve not only H_2O_2 decomposition but also hydroxyl radical ($^{\cdot}OH$) generation with a superoxide anion ($O_2^{\cdot-}$) and H_2O_2 as substrates (Schopfer et al., 2001). Experimental evidence suggests that $^{\cdot}OH$ is involved in pollen wall loosening (at the germination aperture) whereas H_2O_2 strengthens the wall through peroxidase-mediated ferulate oxidation in tobacco pollen (Smirnova et al., 2014).

Mentioned above, several studies on the activity of peroxidases implicated in physiological processes of pollen had been described, the involvement in the regulation of cell wall extensibility during germination, and its increased activity observed during maturation suggests a crucial role of peroxidase in pollen development. Thus, monitoring changes in peroxidase activity can provides information on germinability of pollen. However, there is little known the effect of difference in storage conditions affecting pollen viability on the activity of peroxidase in kiwifruit pollen.

It is known that hydrolytic enzymes which diffuse from pollen after landing on the receptive stigma could function in pollen tube growth and penetration (Knox, 1984). Some morphogenetic changes that occur during plant development require the disruption of existing plant tissues and it may occur during pollen tube growth through stylar transmitting tissue (Neale et al., 1990). Accumulation of chitinases in bean abscission zone and their presence in anthers and pistils imply that the involvement of chitinases in many plant processes requiring cell wall disruptions in rapidly growing tissues such as young leaves, stems, flowers, and pollen (del Campillo and Lewis, 1992). These observations are further supported by evidence that a high expression of poplar *win*6 gene (wound-inducible chitinase) was noted

# These authors contributed equally to this work.
* Corresponding author

in unwounded young leaves, and a sharp increase in *win*6 expression in pollen coincided with the onset of anther dehiscence (CLARKE et al., 1994). However, little is known the major function of chitinase on the viability of kiwifruit pollen, and it remains unclear whether difference in enzyme activity and isozyme patterns is due to changes in pollen viability.

Thus, the objective of this study was to examine the activity and isozyme patterns of peroxidase and chitinase to establish a reliable method for evaluating pollen vigor in kiwifruit. To achieve this objective, we designed the following experiments: 1) under different storage conditions, *in vitro* germination of kiwifruit pollen was tested; 2) through various experimental approaches (*in vitro* germination, active staining, and Congo Red decoloration), whether difference in storage conditions that affected pollen viability was associated with changes in activity of peroxidase was investigated; 3) changes in chitinase activity and isozyme pattern were also investigated under different storage conditions.

Materials and methods

Materials

Kiwifruit pollen grains, *Actinidia chinensis* (Matua I) and *A. chinensis* (Matua II) were purchased from local market in Naju, Jeonnam, Korea and stored in tightly sealed containers with desiccant at -20 °C until further use.

Germination of kiwifruit pollen

Unless otherwise noted, kiwifruit pollen cultivar Matua I was used for *in vitro* germination. Prior to germination, stored pollen was rehydrated for 12 h at 4 °C in a sealed container lined with moist filter paper. The pollen was scattered uniformly on a medium containing 10% sucrose (w/v) and 1% agar (w/v) and then incubated in the dark at 25 °C with a relative humidity (RH) of 80-90%. To determine difference in pollen viability by storage conditions, pollen was subjected to the following storage conditions: heat-treated frozen storage (HF), fresh frozen storage (FF), moisture-treated frozen storage (MF), moisture-treated cold storage (MC), and moisture-treated RT storage (MR). For HF preparation, pollen was heated at a temperature of 100 °C using a moisture analyzer (MB23, OHAUS Co., USA) with infrared heat source for 10 min and then kept at -20 °C for 24 h. For FF preparation, pollen was kept at -20 °C and no treatment was applied until use. For MF, MC, and MR preparations, pollen was placed in a sealed container lined with moist filter paper and stored at 4 °C for 12 h. After that, pollen was subdivided into three groups and each group was kept at -20 °C or 4 °C or 20 °C, respectively, for 24 h. To investigate the effect of duration of room temperature (RT, 20 °C, RH 50~55%) exposure on pollen germination, pollen was collected at various time periods (6, 12, 18, and 24 h) after exposure to RT. Subsequently, germination was measured every 1 h during incubation. In some experiments, either fresh or heat-treated pollen were incubated on a medium supplemented with 0.01% (w/v) Congo Red (Sigma, St. Louis, MO, USA). In other experiments, different contents (0, 10, and 20 nmol) of *N*-acetyl glucosamine (GlcNAc) were added to the germination medium. For pollen germination analysis, digital images of randomly chosen fields were acquired with a video camera (Axiocam ICc1, Carl Zeiss, Germany) connected to Axiostar Plus (Carl Zeiss, Germany). The percentage of germination was determined by scoring at least 50 pollen grains per treatment from digital images. Pollen grains with tube length equal to or exceeding their diameter were considered to have germinated.

Extraction of proteins

Proteins were extracted from kiwifruit pollen (10 mg) according to the method described by PERSIA et al. (2008) with some modifications. Briefly, pollen was suspended in 200 μL of wash buffer (50 mM Tris-HCl, pH 8.5, 3 mM EGTA, 3 mM EDTA, 2 mM $MgCl_2$, and 2 mM dithiothreitol) and incubated at 4 °C for 30 min with occasional shaking. The suspension was centrifuged at 10000 × *g* for 5 min at 4 °C, and the resulting supernatant was collected and kept at -20 °C until use. The pellet was resuspended in 200 μL lysis buffer (50 mM Tris HCl at pH 8.5, 3 mM EGTA, 3 mM EDTA, and 2 mM $MgCl_2$) and homogenized for 2 min on ice using a grinding pestle (BioMasher-II, NIPPI Inc., Tokyo, Japan). The homogenate was kept at 4 °C for 30 min and centrifuged at 10000 × *g* for 5 min at 4 °C, and the resulting supernatant was collected. Crude enzymes obtained from extraction by using wash buffer and lysis buffer, respectively, were used in active staining of chitinase. For POD activity assay, 10 mg of pollen was homogenized in 200 μL of lysis buffer (50 mM Tris-HCl, pH 8.5, 3 mM EGTA, 3 mM EDTA, and 2 mM $MgCl_2$) for 2 min on ice using a grinding pestle (BioMasher-II, NIPPI Inc., Tokyo, Japan). Following incubation at 4 °C for 30 min, the homogenate was centrifuged at 10000 × *g* for 5 min at 4 °C. The resulting supernatant was collected and used as crude extract to determine POD activity. Protein concentration in crude extracts was determined according to the method of BRADFORD (1976) with bovine serum albumin as standard.

Assay of peroxidase activity

POD activity was assayed based on the method described by CHANCE and MAEHLY (1955). The reaction mixture contained 50 μL of 20 mM guaiacol, 2.87 mL of 10 mM phosphate buffer (pH 7.0), and 25 μL of crude extract. The reaction was initiated by adding 20 μL of 40 mM H_2O_2. Increase in absorbance at 470 nm was measured at 1 min intervals using a UV-visible spectrophotometer (Optizen 3220UV, Mecasys Co., Korea). One enzyme unit was defined as the amount of enzyme capable of producing 1 μmol of tetraguaiacol per minute at 25 °C.

Active staining of peroxidase

For in-gel peroxidase activity assay, native polyacrylamide gel electrophoresis (PAGE) was performed according to the method described by ORNSTEIN (1964). Duplicate sets of protein samples (30 ug/lane) (heat-treated Matua I, moisture-treated Matua I, fresh Matua I, and fresh Matua II) were loaded onto each half of a 10% (w/v) gel and resolved by native PAGE. After electrophoresis, gel was divided in half longitudinally and one half was stained with 0.12% (w/v) Coomassie brilliant blue (CBB) R-250. The other half was equilibrated with 50 mM Tris buffer (pH 6.8) for 10 min. The gel was then incubated in 50 mM Tris buffer (pH 6.8) containing 0.46% (v/v) guaiacol and 13 mM H_2O_2 until reddish-brown bands appeared, after which it was fixed in a mixture of water/methanol/acetic acid (6.5:2.5:1, v/v) as described previously (CARUSO et al., 1999).

Active staining of chitinase

Chitinase active staining was performed according to the method described by TRUDEL and ASSELIN (1989). Crude proteins (5 ug) were loaded into each lane of two different 10% (w/v) gels with or without 0.01% (w/v) glycol chitin embedded, and two gels were resolved in parallel by SDS-PAGE. Following electrophoresis, one gel was stained with 0.12% (w/v) CBB R-250. The other gel was incubated with 100 mM sodium acetate buffer (pH 5.0) containing 1% (v/v) Triton X-100 and 1% (w/v) skim milk at 37 °C for 2 h with reciprocal shaking. This was followed by overnight incubation under the same conditions except that there was no skim milk in the buffer solution. Subsequently, the gel was immersed in 500 mM Tris-HCl (pH 8.9) solution containing 0.01% (w/v) Calcofluor white M2R (Sigma, St.

Louis, MO, USA) for 7 min, and lysed zones were visualized and photographed using a UV transilluminator (WGD-30; Daihan Sci. Co., Korea).

Statistical analysis

Data were compared using Tukey's Studentized range (HSD) test, with $P \leq 0.05$ indicating statistical significance. All data were analyzed using Statistical Analysis System 9.1 and are presented as mean ± standard error (SE).

Results and discussion

Germination of kiwifruit pollen at various storage conditions

To identify change in pollen performance after storage under different conditions, germinations of kiwifruit pollen subjected to five storage conditions mentioned above were evaluated. As shown in Fig. 1A, a germination of 72.3% was observed in FF treatment, while little (0.9%) or no germination was observed in HF treatment.

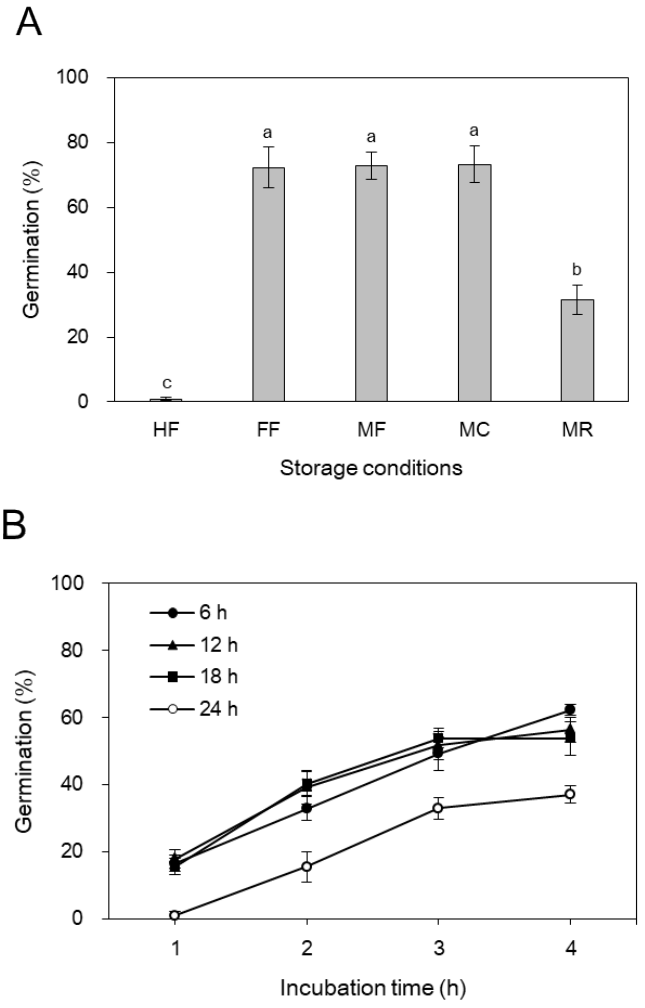

Fig. 1: Effects of different storage conditions on *in vitro* germination of kiwifruit pollen.
Pollen germination was observed following five storage conditions (A) or after various durations of RT exposure (B). HF, heat-treated frozen storage; FF, fresh frozen storage; MF, moisture-treated frozen storage; MC, moisture-treated cold storage; MR, moisture-treated RT storage. Different letters on the error bars indicate significant differences based on Tukey's Studentized range at $p \leq 0.05$. Error bars represent the SE of mean values ($n = 6$).

The germination of MF or MC treatment was similar to that of FF treatment, while that of MR treatment exhibited a 2.3-fold decrease. These results indicate that storage at high temperature has a negative impact on pollen germination while moisture pre-treatment has no effect on pollen germination. A previous report has shown that prolonged exposure to high humidity (95% RH for 5 h) at 38 °C does not affect the viability of tobacco pollen as assessed by fluorochromatic reaction, although it significantly reduces pollen germination *in vitro* (Shivanna and Cresti, 1989). When pollen grains were subjected to both high RH and high temperature simultaneously, their germination was delayed compared to that of pollen exposed to high RH at ambient temperature *vitro* (Shivanna and Cresti, 1989). This is in agreement with our observations. In the present study, we found that pollen germination of MR treatment was significantly lower than that of moisture treated-group (MF or MC) though their temperature conditions were more favorable for germination than those tested by Shivanna and Cresti (1989).

The effect of temperature was also evident when germination of kiwifruit pollen was examined after RT exposure for various durations (6, 12, 18, and 24 h). We found that germinations were similar when kiwifruit pollen was exposed to RT ranging from 6 to 18 h. However, when exposure was more than 18 h, pollen germination reduced to <40% (Fig. 1B). These results indicate that duration of RT exposure above 18 h has adverse effect on pollen germination.

Deng and Harbaugh (2004) have investigated the effect of storage temperature (RT, 4 °C, and -20 °C) over a period of 6 days on caladium pollen viability. Their results showed that only 5% of pollen stored at RT for 1 day remained viable. More than 99% of pollen lost their viability after being stored at -20 °C for 1 day. In contrast, storage temperature of 4 °C seemed to slow down the decline of germination and it has resulted in 32.5% viability after 1 day. The decrease in pollen viability under RT storage has also been described elsewhere (Borghezan et al., 2011; Novara et al., 2017; Mesnoua et al., 2018; Yuan et al., 2018), showing that pollen stored at subzero temperature is effective for long-term preservation.

Peroxidase activity of kiwifruit pollen

We have previously observed that the germination of kiwifruit pollen varied with storage conditions. We also confirmed that pollen germination in MR treatment decreased by less than a half compared to those in other conditions except HF treatment. To determine whether changes in activity and isozyme patterns of POD and chitinase were due to difference in storage conditions affecting viability of kiwifruit pollen, the following experiments were conducted under the same storage conditions tested above. First, we investigated POD activity in kiwifruit pollen using various experimental approaches. As shown in Fig. 2A, POD activity of kiwifruit pollen increased when proportions of viable pollen were increased. There was a positive correlation between pollen viability and POD activity (R^2=0.993), suggesting that POD activity could be used as a valuable index to predict pollen viability. A comparison of POD activity in kiwifruit pollen under different storage conditions showed that POD activity was relatively lower in HF treatment than that in any other group (Fig. 2B). In HF treatment, POD activity was observed. However, pollen grains were non-viable. This is consistent with our previous result showing high level of POD activity in HF-treated pear pollen (Song et al., 2018). This might be partially attributed to residual activities caused by the presence of POD enzymes in non-viable pollen (Rodriguez-Riano and Dafni, 2000). Although the reduction in POD activity was not drastic in other treatments except for HF treatment, these results indicate that it is most suitable to store kiwifruit pollen under FF treatment to maintain the pollen vigor. POD activities of two kiwifruit cultivars (Matua I and II) showed similar levels (Fig. 2C).

POD activity of kiwifruit pollen was also confirmed by active

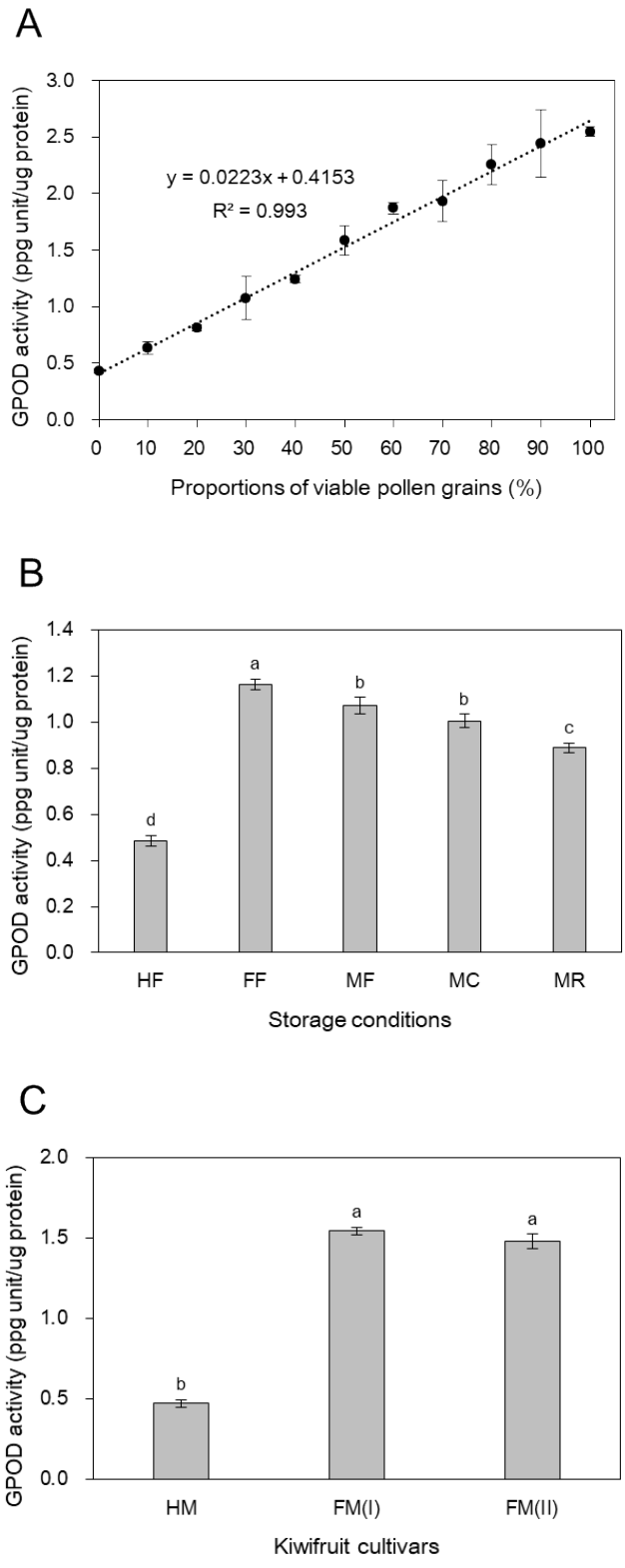

Fig. 2: Changes in peroxidase (POD) activity of kiwifruit pollen. The activity of POD was examined based on proportions of viable pollen grains (A), under different storage conditions (B), and between kiwifruit cultivars (Matua I and II) (C). HF, heat-treated frozen storage; FF, fresh frozen storage; MF, moisture-treated frozen storage; MC, moisture-treated cold storage; MR, moisture-treated RT storage; HM, heat-treated Matua I; FM(I), fresh Matua I; FM(II), fresh Matua II. Different letters on error bars indicate significant differences based on Tukey's Studentized range at $p \leq 0.05$. Error bars represent the SE of mean values ($n = 3$).

staining (Fig. 3). POD enzymes were seen as yellow to dark yellow bands on a clear background (Fig. 3B). Two isoform bands at around 66 kDa were found, and there was a detectable difference in the relative activity of these two isoforms among treatments. The staining intensity of moisture-treated pollen was slightly lower than those of fresh pollens (Fig. 3B, compare lane B with lanes C and D). In the case of heat-treated pollen, there was a very faint band that was easily distinguishable compared with those of other treatments (Fig. 3B, lane A).

Peroxidase isozyme profiles of pollen collected before and after anthesis have been investigated (Parui et al., 1998). Considerable variation in the enzyme profiles was observed, with individual isoforms showing a tendency to increase in their staining intensity upon maturing of anther. The increase in activity of peroxidase isoenzymes with maturity of pollen can be explained by the fact that peroxidases play an important role in cell wall biosynthesis (Passardi et al., 2004). Furthermore, observed changes in the activity of peroxidase isozymes during pollen germination have been confirmed in a previous study of Malik and Gupta (1976).

Dai et al. (2007) have screened 186 protein spots differentially expressed in mature and germinated rice pollen using 2-DE-based proteomic approaches. Their study revealed that wall metabolism-

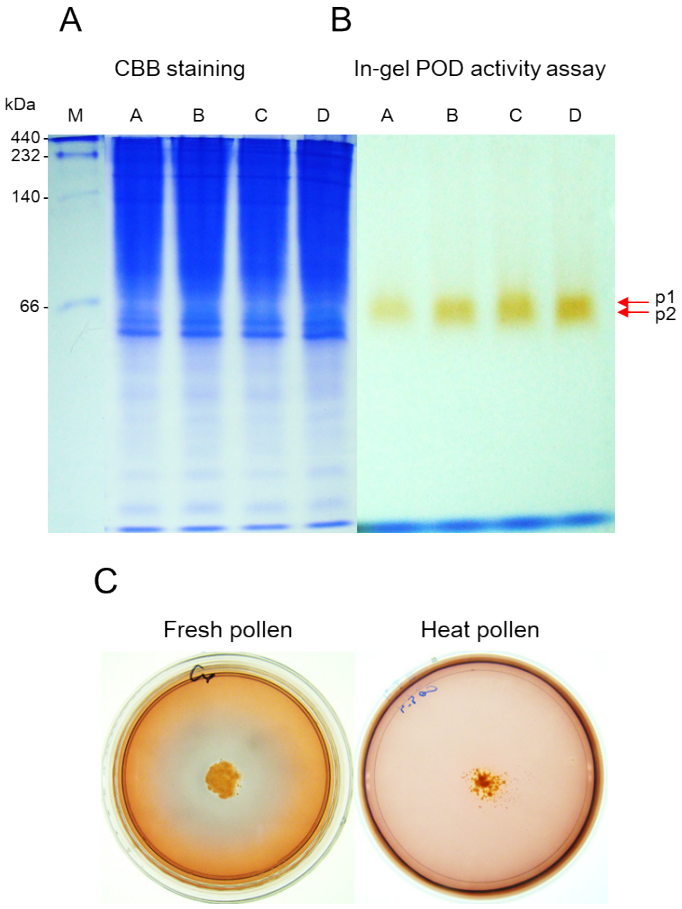

Fig. 3: Identification of POD activity in kiwifruit pollen. Proteins were loaded to 10% (w/v) gel and resolved by native PAGE. After electrophoresis, protein bands were visualized by CBB staining (A), and POD activities were detected by in-gel activity assay (B). POD activity was also confirmed by decoloration of germination medium supplemented with 0.01% (w/v) Congo Red (C). M, size marker; A, heat-treated Matua I; B, moisture-treated Matua I; C, fresh Matua I; D, fresh Matua II. Arrows indicate two POD isoforms.

related proteins such as UDP-glucose pyrophosphorylase, polygalacturonase, class III peroxidases, and β-expansins were up-regulated on germination, might be mainly responsible for pollen tube growth. This study also found that there were multiple isoforms in differentially expressed proteins and different expression patterns between isoforms during the developmental switch from mature pollen to germination and tube growth. In the case of β-expansin and class III peroxidase, some isoforms were up-regulated and others were down-regulated, suggesting that they involved differently in pollen germination.

As mentioned in the introduction, plant peroxidases catalyze reactions involving not only H_2O_2 decomposition but also $^{\cdot}OH$ generation with superoxide anion ($O_2^{\cdot-}$) and H_2O_2 as substrates (SCHOPFER et al., 2001). Nitrogen double bonds that are characteristic of Azo dyes (such as Congo Red) may be broken via oxidation by $^{\cdot}OH$ generated from peroxidase, thereby resulting in the loss of color (CRIPPS et al., 1990). Interestingly, during the early stage of kiwifruit pollen germination, extracellular H_2O_2 accumulation in the culture medium by germinating pollen has been reported (SPERANZA et al., 2012). Thus one can assume, based on the results presented by SPERANZA et al. (2012), that it is possible to judge pollen vigor depending on the degree of decoloration of the medium through POD activity when kiwifruit pollen is cultured in a germination medium containing Congo Red. To determine whether the degree of decoloration might be different depending on pollen vigor, fresh or heat-treated pollen were incubated on a medium supplemented with Congo Red. As shown in Fig. 3C, a measurable decoloration (more than half of the diameter) was observed in germination medium with which fresh pollen was cultured. However, no decoloration was observed in the germination medium with which heat-treated pollen was cultured. Some metabolites such as $^{\cdot}OH$ generated by fresh pollen harboring POD activity during germination might have contributed to the observed decoloration in germination medium. Fungal lignin peroxidase and plant horseradish peroxidase are known to be involved in the decoloration of Azo dyes (CRIPPS et al., 1990). Our finding, together with previously discussed results (that is, change of POD activity with different storage conditions, correlation between pollen viability and POD activity, active staining of POD enzyme, and the decoloration of germination medium), suggest that monitoring POD activity can help verify pollen performance in kiwifruit.

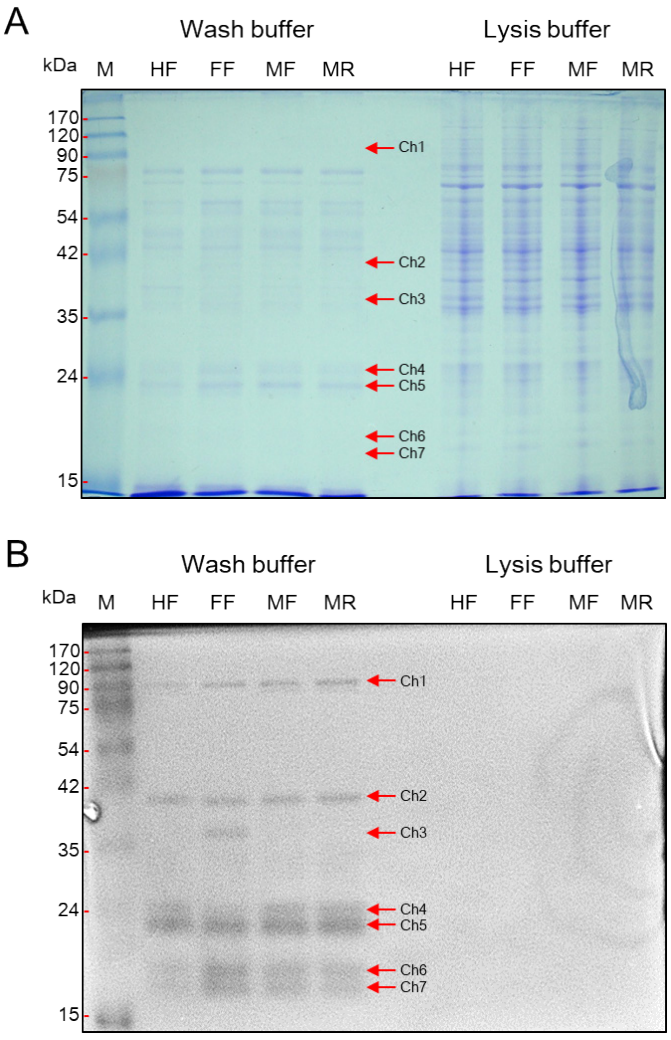

Fig. 4: Changes in activity and isozyme pattern of chitinase in kiwifruit pollen.
Crude proteins were extracted by using wash buffer or lysis buffer and loaded into each lane of two different 10% (w/v) gels with or without 0.01% (w/v) glycol chitin embedded. Following electrophoresis, protein bands were visualized by CBB staining (A), and individual chitinase isozymes were detected by chitinase active staining (B). M, size marker; HF, heat-treated frozen storage; FF, fresh frozen storage; MF, moisture-treated frozen storage; MR, moisture-treated RT storage. Arrows indicate seven chitinase isoforms.

Chitinase activity of kiwifruit pollen

To evaluate the potential of chitinase enzyme as an indicator for pollen vigor, chitinase activity in kiwifruit pollen was examined under different storage conditions (HF, FF, MF, and MR). As shown in Fig. 4B, seven isozymes (Ch1-Ch7) were detected, and the relative molecular weights of these enzymes were found to differ, ranging from 15 to 90 kDa through active staining. For some isozymes, there were detectable differences in the relative activity among treatments. Staining intensities of Ch6 and Ch7 were quite higher in FF treatment than those in other treatments. Interestingly, Ch3 isoform was detected only in FF treatment among all seven isoforms. We found that some isozymes such as Ch2, Ch4, and Ch5 were detected in all treatments. In contrast, isozyme Ch6 or Ch7 was not detected in HF treatment. These results indicate that activities of individual chitinase isozymes present in the kiwifruit pollen can differ depending on storage conditions which have a direct impact on pollen vigor. Although direct evidence showing that chitinase isozymes are implicated in pollen vigor is still uncertain, monitoring Ch3 isoform may help verify the performance of kiwifruit pollen.

Plant chitinases (EC 3.2.1.14) are known to protect plants against fungal pathogens by inhibiting mycelial growth or by degrading chitin, a linear homopolymer of β-1,4-linked *N*-acetyl glucosamine (GlcNAc) residue (COLLINGE et al., 1993). The possible involvement of chitinase has been elucidated in non-defensive roles such as flowering, reproduction, seed germination, somatic embryogenesis, and plant growth (PATIL and WIDHOLM, 1997). Furthermore, differential regulation of individual chitinase isoforms in response to pathogen attacks and abiotic stresses has been reported in peas, barley, tobacco, and peanut (COLLINGE et al., 1993). These findings motivated us to further investigate the potential involvement of chitinase in pollen germination. Hence, we studied whether pollen germination was affected by *in vitro* supplement of GlcNAc. To assess the effect of GlcNAc, kiwifruit pollen was incubated in germination medium supplemented with either 0 (control), 10, or 20 nmol GlcNAc. As shown in Fig. 5, treatment with 10 or 20 nmol of GlcNAc gave rise to pollen germination of 77.1% or 74.5%, respectively, compared to germination of 60.3% in the non-treated group. Although the addition of GlcNAc did not show a dose-dependent effect, our results implied a possible involvement of chitinase in kiwifruit pollen germination. Endogenous substrate for plant chitinases has not yet been found.

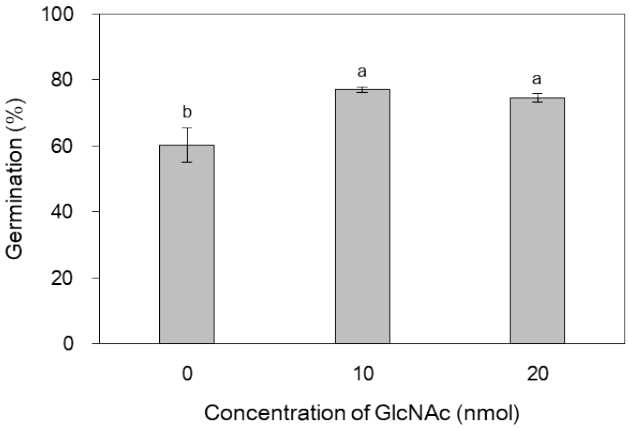

Fig. 5: Effect of GlcNAc on *in vitro* germination of kiwifruit pollen. Pollen was incubated in germination medium supplemented with varying amounts (0, 10, or 20 nmol) of GlcNAc. Different letters on the error bars indicate significant differences based on Tukey's Studentized range at $p \leq 0.05$. Error bars represent SE of mean values ($n = 3$).

However, there is strong evidence that these enzymes can catalyze hydrolytic decomposition of arabinogalactan proteins (AGPs) present in plant (VAN HENGEL et al., 2001). VAN HENGEL et al. (2001) have shown that immature carrot seed AGPs that can promote somatic embryogenesis contain GlcNAc and glucosamine residues. In addition, they are sensitive to endochitinase cleavage. AGPs are commonly found in stigma exudates, transmitting tissues, pollen grains, and pollen tubes in many species. They have been implicated in pollen formation and hydration as well as in pollen tube growth and guidance (NGUEMA-ONA et al., 2012). In tobacco, stylar transmitting tissue-specific glycoproteins belonging to AGP family are implicated in promoting pollen tube growth and affecting pollen tube guidance, and its sugar moieties may be a source of nutrients for these biological activities (CHEUNG et al., 1995; WU et al., 1995). Thus, it can be inferred that chitinases present in pollen may also participate in pollen tube growth through hydrolytic action on endogenous substrate.

Conclusion

This study shows that the viability of kiwifruit pollen differs depending on storage conditions and that storing pollen in FF treatment is the most desirable for maintaining pollen vigor. POD activity was found to be closely related to pollen viability. Although residual POD activity was detected in heat-treated pollen, POD activity was also altered by different storage conditions as revealed by preliminary studies in which pollen viability was affected depending on storage conditions. These results (i.e., detectable difference in relative activity of POD enzymes between heat-treated and viable pollen, and decoloration of Congo Red caused by fresh pollen possessing POD activity) demonstrate that monitoring POD activity can help verify pollen performance in kiwifruit. Furthermore, distinction of chitinase isoforms may facilitate more precise identification of viable pollen which possesses germination potential from non-viable pollen. Taken together, monitoring the activity of POD and chitinase can be an attractive alternative to evaluate pollen vigor in kiwifruit.

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References

ASHFORD, A., KNOX, R., 1980: Characteristics of pollen diffusates and pollen wall cytochemistry in poplars. J. Cell Sci. 44, 1-17.

BORGHEZAN, M., CLAUMAN, A.D., STEINMACHER, D.A., GUERRA, M.P., ORTH, A.I., 2011: *In vitro* viability and preservation of pollen grain of kiwi (*Actinidia chinensis* var. *deliciosa* (A. Chev.) A. Chev). Crop Breed. Appl. Biotechnol. 11, 338-344. DOI: 10.1590/S1984-70332011000400007

BRADFORD, M.M., 1976: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254. DOI: 10.1016/0003-2697(76)90527-3

BREDEMEIJER, G., 1974: Peroxidase activity and peroxidase isoenzyme composition in self-pollinated, cross-pollinated and unpollinated styles of *Nicotiana alata*. Acta Bot. Neerl. 23, 149-157. DOI: 10.1111/j.1438-8677.1974.tb00931.x

BREDEMEIJER, G., 1984: The role of peroxidases in pistil-pollen interactions. Theor Appl. Genet. 68, 193-206. DOI: 10.1007/BF00266889

CARUSO, C., CHILOSI, G., CAPORALE, C., LEONARDI, L., BERTINI, L., MAGRO, P., BUONOCORE, V., 1999: Induction of pathogenesis-related proteins in germinating wheat seeds infected with *Fusarium culmorum*. Plant Sci. 140, 87-97. DOI: 10.1016/S0168-9452(98)00199-X

CHANCE, B., MAEHLY, A., 1955: Assay of catalases and peroxidases. Methods Enzymol. 2, 764-775. DOI: 10.1016/S0076-6879(55)02300-8

CHAUDHARY, G., DANTU, P.K., KAYASTHA, A.M., 2010: Differential Activity of four Selected Enzymes in the Pistils of two Members of the Family Bignoniaceae. J. Proteins Proteom. 1, 71-76.

CHEUNG, A.Y., WANG, H., WU, H.M., 1995: A floral transmitting tissue-specific glycoprotein attracts pollen tubes and stimulates their growth. Cell 82, 383-393. DOI: 10.1016/0092-8674(95)90427-1

CLARKE, H.R., DAVIS, J.M., WILBERT, S.M., BRADSHAW, H.D., GORDON, M.P., 1994: Wound-induced and developmental activation of a poplar tree chitinase gene promoter in transgenic tobacco. Plant Mol. Biol. 25, 799-815. DOI: 10.1007/BF00028875

COLLINGE, D.B., KRAGH, K.M., MIKKELSEN, J.D., NIELSEN, K.K., RASMUSSEN, U., VAD, K., 1993: Plant chitinases. Plant J. 3, 31-40. DOI: 10.1046/j.1365-313X.1993.t01-1-00999.x

CRIPPS, C., BUMPUS, J.A., AUST, S.D., 1990: Biodegradation of azo and heterocyclic dyes by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 56, 1114-1118.

DAFNI, A., FIRMAGE, D., 2000: Pollen viability and longevity: Practical, ecological and evolutionary implications. Plant Syst. Evol. 222, 113-132. DOI: 10.1007/BF00984098

DAFNI, A., MAUÉS, M.M., 1998: A rapid and simple procedure to determine stigma receptivity. Sex. Plant Reprod. 11, 177-180. DOI: 10.1007/s004970050138

DAI, S., CHEN, T., CHONG, K., XUE, Y., LIU, S., WANG, T., 2007: Proteomics identification of differentially expressed proteins associated with pollen germination and tube growth reveals characteristics of germinated *Oryza sativa* pollen. Mol. Cell. Proteomics 6, 207-230. DOI: 10.1074/mcp.M600146-MCP200

DASHEK, W.V., ERICKSON, S.S., HAYWARD, D.M., LINDBECK, G., MILLS, R.R., 1979: Peroxidase in cytoplasm and cell wall of germinating lily pollen. Bot. Gaz. 140, 261-265.

DEL CAMPILLO, E., LEWIS, L.N., 1992: Occurrence of 9.5 cellulase and other hydrolases in flower reproductive organs undergoing major cell wall disruption. Plant Physiol. 99, 1015-1020. DOI: 10.1104/pp.99.3.1015

DENG, Z., HARBAUGH, B.K., 2004: Technique for in vitro pollen germination and short-term pollen storage in caladium. HortScience 39, 365-367.

KNOX, R., 1984: Pollen-pistil interactions. In: Linskens, H.F., Heslop-Harrison, J. (eds.), Cellular interactions, 508-608. Berlin, Heidelberg, Germany, Springer-Verlag. DOI: 10.1007/978-3-642-69299-4_24

MALIK, C., GUPTA, S., 1976: Changes in peroxidase isoenzymes during germination of pollen. Biochem. Physiol. Pflanz. 169, 519-522. DOI: 10.1016/S0015-3796(17)30903-4

Mesnoua, M., Roumani, M., Salem, A., 2018: The effect of pollen storage temperatures on pollen viability, fruit set and fruit quality of six date palm cultivars. Sci. Hortic. 236, 279-283. DOI: 10.1016/j.scienta.2018.03.053

Neale, A.D., Wahleithner, J.A., Lund, M., Bonnett, H.T., Kelly, A., Meeks-Wagner, D.R., Peacock, W.J., Dennis, E.S., 1990: Chitinase, β-1,3-glucanase, osmotin, and extensin are expressed in tobacco explants during flower formation. Plant Cell 2, 673-684. DOI: 10.1105/tpc.2.7.673

Nguema-Ona, E., Coimbra, S., Vicré-Gibouin, M., Mollet, J.C., Driouich, A., 2012: Arabinogalactan proteins in root and pollen-tube cells: distribution and functional aspects. Ann. Bot. 110, 383-404. DOI: 10.1093/aob/mcs143

Novara, C., Ascari, L., La Morgia, V., Reale, L., Genre, A., Siniscalco, C., 2017: Viability and germinability in long term storage of *Corylus avellana* pollen. Sci. Hortic. 214, 295-303. DOI: 10.1016/j.scienta.2016.11.042

Ornstein, L., 1964: Disc electrophoresis-i background and theory. Ann. N. Y. Acad. Sci. 121, 321-349. DOI: 10.1111/j.1749-6632.1964.tb14207.x

Pandey, V.P., Awasthi, M., Singh, S., Tiwari, S., Dwivedi, U.N., 2017: A comprehensive review on function and application of plant peroxidases. Biochem. Anal. Biochem. 6, 308. DOI: 10.4172/2161-1009.1000308

Parui, S., Mondal, A.K., Mandal, S., 1998: Peroxidase isozyme profiles of immature and mature pollen of seven tropical plants from eastern India. Grana 37, 228-232. DOI: 10.1080/00173139809362671

Passardi, F., Penel, C., Dunand, C., 2004: Performing the paradoxical: how plant peroxidases modify the cell wall. Trends Plant Sci. 9, 534-540. DOI: 10.1016/j.tplants.2004.09.002

Patil, V., Widholm, J., 1997: Possible correlation between increased vigour and chitinase activity expression in tobacco. J. Exp. Bot. 48, 1943-1950. DOI: 10.1093/jxb/48.11.1943

Persia, D., Cai, G., Del Casino, C., Faleri, C., Willemse, M.T., Cresti, M., 2008: Sucrose synthase is associated with the cell wall of tobacco pollen tubes. Plant Physiol. 147, 1603-1618. DOI: 10.1104/pp.108.115956

Rodriguez-Riano, T., Dafni, A., 2000: A new procedure to asses pollen viability. Sex. Plant Reprod. 12, 241-244. DOI: 10.1007/s004970050008

Schopfer, P., Plachy, C., Frahry, G., 2001: Release of reactive oxygen intermediates (superoxide radicals, hydrogen peroxide, and hydroxyl radicals) and peroxidase in germinating radish seeds controlled by light, gibberellin, and abscisic acid. Plant Physiol. 125, 1591-1602. DOI: 10.1104/pp.125.4.1591

Shivanna, K., Cresti, M., 1989: Effects of high humidity and temperature stress on pollen membrane integrity and pollen vigour in *Nicotiana tabacum*. Sex. Plant Reprod. 2, 137-141. DOI: 10.1007/BF00192759

Smirnova, A., Matveyeva, N., Yermakov, I., 2014: Reactive oxygen species are involved in regulation of pollen wall cytomechanics. Plant Biol. 16, 252-257. DOI: 10.1111/plb.12004

Song, Y., Lee, S., Jo, J., Choi, S., Seo, D., Wi, S., Yang, U., Jung, W., 2018: Changes in the activity of peroxidase and dehydrogenase in pear pollen during germination under different storage conditions. Hortic. Sci. Technol. 36, 777-788. DOI: 10.12972/kjhst.20180076

Speranza, A., Crinelli, R., Scoccianti, V., Geitmann, A., 2012: Reactive oxygen species are involved in pollen tube initiation in kiwifruit. Plant Biol. 14, 64-76. DOI: 10.1111/j.1438-8677.2011.00479.x

Trudel, J., Asselin, A., 1989: Detection of chitinase activity after polyacrylamide gel electrophoresis. Anal. Biochem. 178 (2), 362-366. DOI: 10.1016/0003-2697(89)90653-2

van Hengel, A.J., Tadesse, Z., Immerzeel, P., Schols, H., Van Kammen, A., de Vries, S.C., 2001: N-acetylglucosamine and glucosamine-containing arabinogalactan proteins control somatic embryogenesis. Plant Physiol. 125, 1880-1890. DOI: 10.1104/pp.125.4.1880

Wu, H.M., Wang, H., Cheung, A.Y., 1995: A pollen tube growth stimulatory glycoprotein is deglycosylated by pollen tubes and displays a glycosylation gradient in the flower. Cell 82, 395-403. DOI: 10.1016/0092-8674(95)90428-X

Yuan, S.C., Chin, S.W., Lee, C.Y., Chen, F.C., 2018: Phalaenopsis pollinia storage at sub-zero temperature and its pollen viability assessment. Bot. Stud. 59, 1. DOI: 10.1186/s40529-017-0218-2

Address of the corresponding author:
Woo-Jin Jung, Department of Agricultural Chemistry, Institute of Environmentally-Friendly Agriculture (IEFA), College of Agriculture and Life Sciences, Chonnam National University, Gwangju 61186, Korea
E-mail address: woojung@jnu.ac.kr