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Optimization of storage temperature for the pollen viability of transgenic plants that express the anti-breast cancer monoclonal antibody mAb BR55

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Abstract

Pollen germination viability is an essential factor in the production of seeds from pollination and fertilization. In this study, we optimized storage temperature conditions to increase the viability of pollen germination and pollen germ tube growth in transgenic plants that express the anti-breast cancer monoclonal antibody mAb BR55. The pollen of the transgenic plant BR55 was stored at five different temperatures: room temperature (RT), 4°C, -20°C, -70°C, or -196°C for 10 weeks. The *in vitro* pollen germination rate and pollen tube growth were observed at 10 weeks. The pollen was then germinated *in vitro* in sucrose buffer, either alone, or supplemented with 20 μg/mL boric acid. In general, both pollen germination rate and pollen tube growth increased with boric acid supplementation, compared to the control. The pollen germination rate and the pollen germ tube length were the greatest in pollen stored at -20°C ($p < 0.01$). The transgenic plant carrying the anti-colorectal cancer mAb CO17-1A *HC* and *LC* genes were pollinated using pollen (mAb BR55 transgenic plant) stored at -20°C, generating F_1 seedlings carrying both mAb CO17-1A and mAb BR55 *HC* and *LC* genes. These results indicate that -20°C is the optimal temperature for pollen storage and ensures the pollen germination viability of transgenic tobacco plants that express therapeutic anti-cancer mAbs.

Keywords: boric acid; breast cancer; monoclonal antibody; pollen germination; storage temperature; transgenic plant.
Abbreviations: HC_heavy chain; LC_light chain; mAb^P_plant-derived monoclonal antibody; mAb^M_mammalian-derived monoclonal antibody.

Introduction

Recent developments in plant biotechnology have facilitated the production of recombinant monoclonal antibodies in plants (mAb^P) as a safe and inexpensive alternative to mammalian-derived monoclonal antibodies (mAb^M) (Gomez et al., 2006; Ko et al., 2003). In addition, each plant that expresses the light chain (LC) or heavy chain (HC) protein subunits of an antibody can easily be crossed to generate F_1 seeds and plant siblings, in which the LC and HC subunits are expressed and assembled. Transgenic plants have already been successfully developed for the production of anti-colorectal cancer mAb^M CO17-1A and anti-breast cancer mAb^M BR55 (Ahn et al., 2007; Brodzik el al., 2006; Ko et al., 2003; Park et al., 2011). If a single transgenic plant was capable of expressing two mAbs for two different antigenic targets, this would significantly enhance the efficiency of mAb production and therapeutic treatment (Jamal et al., 2012). Multiple-mAb plants also have the advantage of lower production costs and higher yields when cultivation space is limited (Jamal et al., 2009). Pollen germination viability and pollen tube growth are important for crossbreeding transgenic plants to express multiple mAbs. Effective pollination and fertilization are required for seed formation (Abdelgadir et al., 2012; Shivanna et al., 1985). The seed can then be used to maintain and safely transfer useful genes to subsequent generations. Furthermore, breeding between siblings from two different parental plants is the most efficient way of generating new varieties (Koga et al., 2004). However, flowering and pollen maturation are often not synchronized between two parental plants that express mAb subunits. Therefore, biologically active pollen should be stored until its counterpart plant is ready for pollination. Low temperatures have been reported to be effective for the long-term preservation of pollen (Parfritt et al., 1984); however, the effect of storage temperature on pollen viability has yet to be studied in transgenic plants. Boric acid is an essential microelement for growth and development in plants (Wang et al., 2003). Boron promotes pollen germination by affecting H^+-ATPase activity, which initiates pollen germination and germ tube growth (Obermeyer et al., 1996). In our previous study, we investigated pollen viability after short-term pollen storage in non-transgenic and transgenic plants (Ahn et al., 2007). In the

present study, the effect of different storage temperatures on the pollen viability of transgenic tobacco plants was examined for long-term storage. The main objective of this study was to determine the ideal temperature for the long-term storage of pollen from transgenic tobacco plants, while maintaining active pollen germination and pollen tube growth.

Results and Discussion

Effect of boric acid on in vitro pollen germination viability

Transgenic tobacco plants were obtained by *Agrobacterium*-mediated transformation using a plant binary vector, pBI BR55K (Kim et al., 2012) (Fig. 1A), which harbored two different promoters, the cauliflower mosaic virus 35S promoter and the Pin2 promoter, to drive expression of the *HC* and *LC* genes, respectively. The pollen was obtained from fully blossomed flowers (Fig. 1B). The effect of boric acid supplementation on *in vitro* pollen germination viability and pollen tube growth was investigated in pollen harvested from transgenic tobacco plants that express anti-breast cancer mAb BR55 (Fig. 1A, expected mAb structure). The results show that in media containing 20 μg/mL boric acid, the pollen germination rate (37%) was significantly higher than the germination rate in media without boric acid (10%) ($p < 0.01$) (Fig. 2A). In addition, pollen tube length was significantly greater with boric acid supplementation (16 nm) than that in media without boric acid (7.4 nm) ($p < 0.01$) (Fig. 2B). Our present data demonstrate that boric acid and storage temperature affect the *in vitro* germination viability of pollen obtained from transgenic tobacco plants that express the anti-breast cancer antibody, mAb BR55. In the present study, boric acid increased pollen germination rate and germ tube growth (Figs. 1 and 2), which confirms earlier findings on pollen germination (Ahn et al., 2006).

Effect of storage temperature on in vitro pollen germination rate

To optimize storage temperature conditions to preserve the pollen of transgenic plants for long-term storage, several temperature conditions (room temperature [RT], 4°C, -20°C, -70°C, and -196°C) were examined for pollen storage. Pollen germination rates and pollen tube growth rates at each storage temperature were analyzed in relation to the presence or absence of boric acid. The results show that pollen stored at -20°C exhibited 180% and 150% higher germination rates without and with boric acid treatment media, respectively, than the other temperature conditions (Fig. 3). RT resulted in the lowest pollen germination rate, regardless of boric acid supplementation. The pollen stored at -196°C exhibited a higher germination rate than pollen stored at -70°C, but a lower rate than pollen stored at -20°C.

Effect of storage temperature on in vitro pollen germ tube growth

Pollen germ tube growth exhibited a response to temperature similar to that of germination. For all the pollen treated with media containing 20 μg/mL boric acid, germ tube growth was lowest for pollen stored at RT ($p < 0.01$) (Fig. 4). Pollen stored at -20°C exhibited the highest germ tube growth rate, regardless of boric acid supplementation (Figs. 4A and 4B).

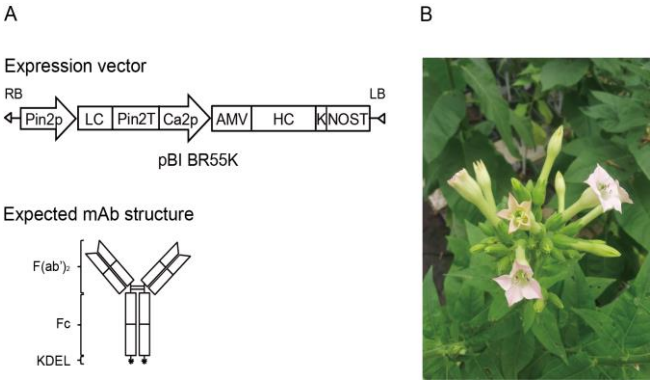

Fig 1. Germination of transgenic plants that express mAb BR55. A. Gene expression vector cassette for *Agrobacterium*-mediated plant transformation. The gene expression cassette had two different promoters (*Pin2p*, a promoter of the proteinase inhibitor II gene from potato, and *Ca2p*, a cauliflower mosaic virus 35S promoter with a duplicated upstream B domain) for the expression of the light chain (LC) and the heavy chain (HC). T1, terminator of the *Pin2* gene from potato; LC and HC, cDNA of the *LC* and *HC* genes of mAb BR55, respectively; AMV, untranslated leader sequence of alfalfa mosaic virus RNA4; T2, terminator of the nopaline synthase (*NOS*) gene; K, KDEL ER retention signal.

Pollen stored at -196°C produced longer germ tubes than that stored at -70°C, but shorter germ tubes than pollen stored at -20°C. Pollen stored at -20°C exhibited significantly higher germ tube growth rate and germination rate than pollen stored at any other temperature, in germination buffer supplemented with boric acid (Fig. 5), whereas pollen stored at -70°C yielded fewer germinated grains and shorter germ tubes compared to pollen stored at -20°C and -196°C (Fig. 5). In general, germination rates and germ tube growth rates were higher in pollen treated with boric acid than in pollen that was grown in media supplemented with sucrose alone. We studied the effect of storage temperature on pollen germination rate and pollen germ tube growth (Figs. 3 and 4, respectively). The effects of different environmental factors on pollen germination and pollen germ tube growth are widely documented in the literature (Dafni et al., 2000; Taylor et al., 1997). It is clear that storage temperature affects germination, by influencing the rate of pollen aging as well as the germination rate (Shivanna et al., 1991; Young et al., 2004). The pollen of some plant species may retain viability for years when stored at extremely low temperatures (Alburquerque et al., 2007; Brown et al., 1991; Sato et al., 1998; Stefani et al., 2000). In the present study, compared to the other storage temperatures examined, a -20°C storage temperature was associated with the highest pollen viability after 10 weeks of storage, which is consistent with the findings of previous studies (Alburquerque et al., 2007; Brown et al., 1991; Sato et al., 1998; Stefani et al., 2000).

PCR confirmation of F_1 seedlings carrying both mAb BR55 and mAb CO17-1A genes

Seeds were harvested from CO17-1A transgenic plants pollinated with the -20°C stored pollens of a BR55 transgenic plant (Fig. 6A). Most F_1 seeds germinated at a rate of 8% (data not shown), and grew to seedlings in Murashige and Skoog

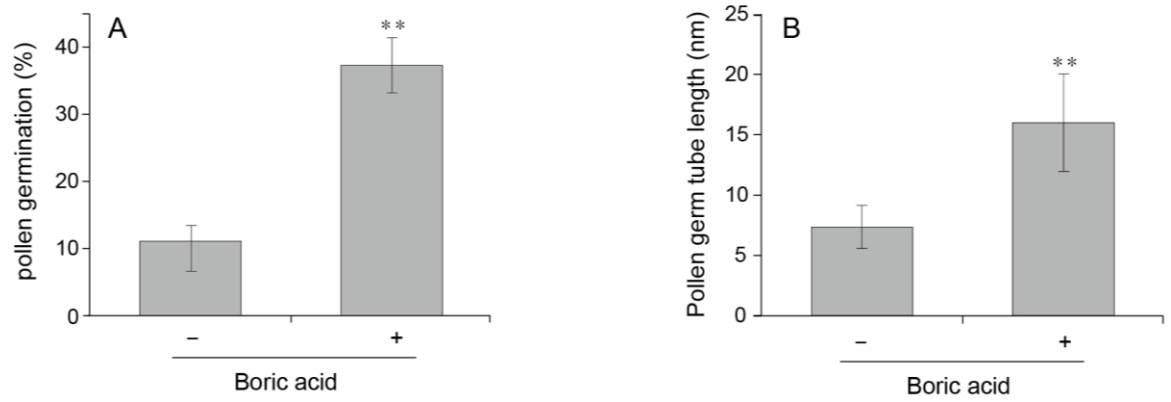

Fig 2. Effect of boric acid on pollen germination (%) and pollen tube growth (%) in transgenic plants. A. Pollen germination rates, with or without 20 μg/mL boric acid, in transgenic plants. B. Pollen tube growth rates, with or without 20 μg/mL boric acid, in transgenic plants. Data represent means and standard errors. Two asterisks indicate significant differences ($p < 0.01$) as analyzed by Student's t-test.

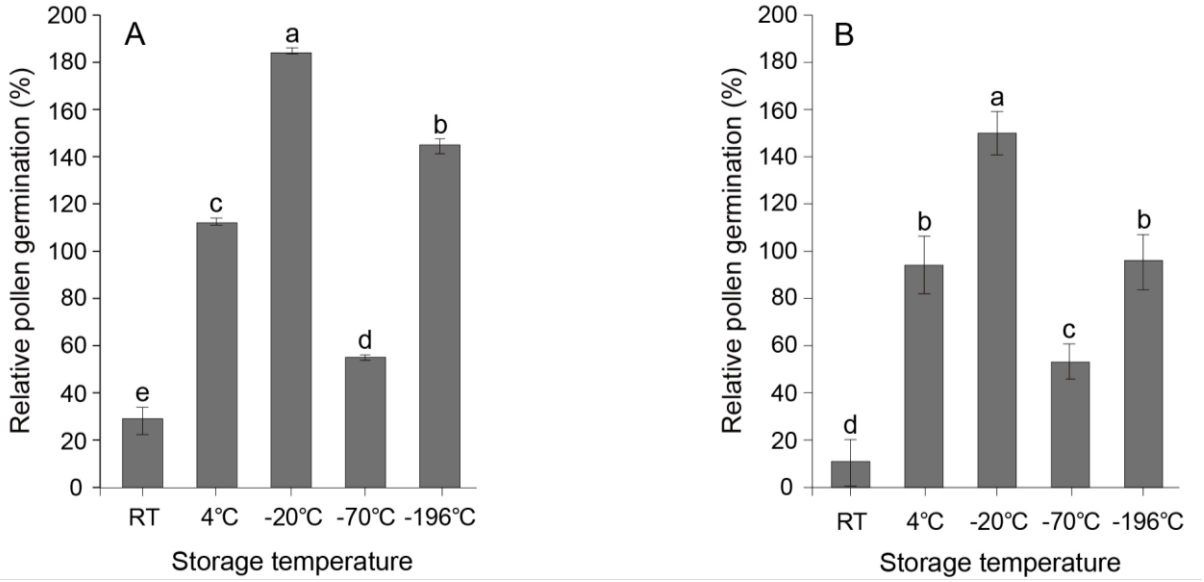

Fig 3. The effect of temperature on relative *in vitro* pollen germination at room temperature (RT), 4°C, -20°C, -70°C, and -196°C in transgenic tobacco plants. A. Pollen germinated in a buffer without boric acid. B. Pollen germinated in a buffer with boric acid. Pollen was stored at RT, 4°C, -20°C, -70°C, or -196°C for 10 weeks. More than 200 pollen grains were scored for each determination. Data represent means and standard errors. Different lowercase letters indicate significant differences ($p < 0.05$) as analyzed by Duncan's multiple range test.

(MS) media containing kanamycin (100 μg/mL). The plant morphology of the F_1 seedlings did not differ from that of the BR55 or CO17-1A parental transgenic plants. PCR amplification using primers for the *HC* and *LC* genes of mAb BR55 and mAb CO17-1A was conducted to confirm their existence in genomic DNA isolated from 13 randomly selected F_1 seedlings grown in kanamycin MS media (Fig. 6B). The mAb CO17-1A *HC* and *LC* gene bands were only detected in seedlings 1, 2, 3, 11, and 12, whereas the mAb BR55 *HC* and *LC* gene bands were only observed in seedlings 4, 9, 10, and 13. Both the mAbs *HC* and *LC* gene bands were detected in seedlings 5, 7, and 8. PCR amplification confirmed the presence of the *HC* and *LC* genes of mAbs BR55 and CO17-1A in the genomic DNA of the F_1 seedlings, indicating that both antibody *HC* and *LC* genes were present in the F_1 transgenic seedlings. Some seedlings exhibited only the mAb CO17-1A or the mAb BR55 genes. It is possible that seedlings 1, 2, 3, 6, 11, and 12, which carried only the mAb CO17-1A genes, might have developed through selfing in the CO17-1A plant itself, and seedlings 4, 9, 10, and 13, which harbored only the mAb BR55 genes, might have been generated through the heterozygosity of the CO17-1A plant. PCR analysis showed that both the mAb CO17-1A and the mAb BR55 *HC* and *LC* genes were present in seedlings 5, 7, and 8. Isolated genomic DNA from the transgenic plants CO and BR was used as a PCR DNA template for the positive controls, whereas the supernatant

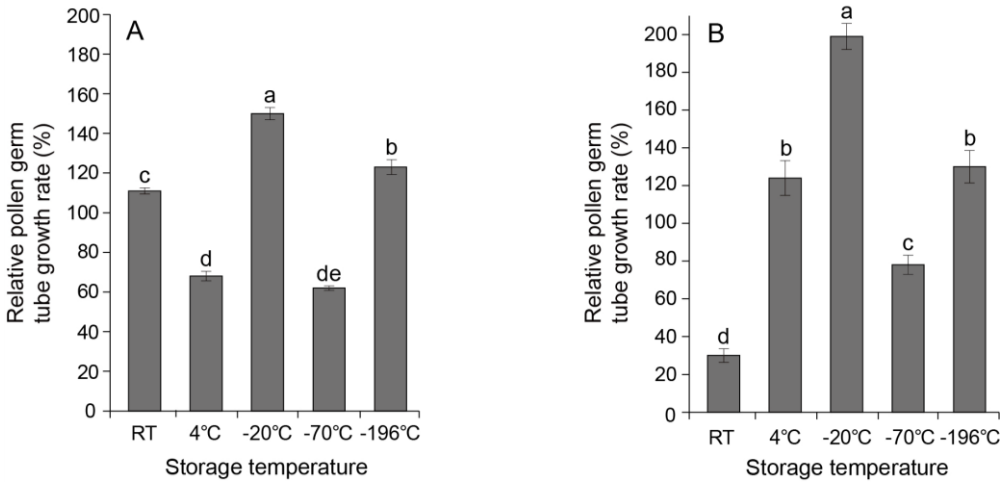

Fig 4. The effect of temperature on relative *in vitro* pollen germ tube growth without (A) and with (B) 20 μg/mL boric acid at room temperature (RT), 4°C, -20°C, -70°C, or -196°C in transgenic tobacco plants. More than 200 pollen grains were scored for each determination. The Figure represents the percentage of pollen germination preserved at the above temperatures. Data represent means and standard errors. Different lowercase letters indicate significant differences ($p < 0.05$) as analyzed by Duncan's multiple range test.

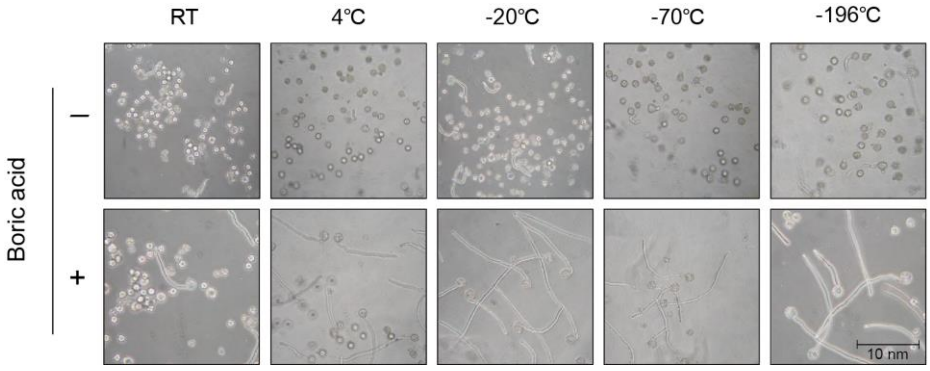

Fig 5. Pollen germ tube growth at different storage temperatures. The pollen of transgenic plants was stored at room temperature (RT), 4°C, -20°C, -70°C, or -196°C. Pollen tube length and pollen germination were higher at -20°C that at the other temperatures, when supplemented with boric acid. Pollen germination and pollen germ tube growth treated with (+) and without (-) 20 μg/mL boric acid were observed under a microscope at low magnification (×100). The bar is 10 nm.

of leaf extract was directly applied to the PCR reaction without genomic DNA isolation in the F_1 seedling samples. Therefore, we suggest that the HC and LC PCR band densities of both the positive controls CO and BR and the transgenic plants were stronger than the PCR band densities of the F_1 seedlings. These results indicate that BR55 pollen stored at -20°C is viable for pollination and fertilization, thus transferring both the *HC* and *LC* genes to the CO17-1A transgenic plant. We therefore conclude that boric acid positively affects pollen germination and germ tube growth in transgenic plants, indicating that it may play an essential role in both of these processes. We also found that storage temperature is an important factor involved in pollen germination rate. Pollen stored at -20°C exhibited the highest rates of growth and germination after 10 weeks of storage, and exhibited high rates of viability for pollination and fertilization. The results of the present study provide useful information regarding transgenic pollen storage conditions, which are essential for the successful breeding of transgenic plants that express multiple therapeutic monoclonal antibodies.

Materials and Methods

Plant and pollen materials

Pollen was obtained from flowers of greenhouse-grown transgenic tobacco plants that express the anti-breast cancer immunotherapeutic, mAb BR55. To produce pollen, transgenic seedlings were obtained from *in vitro* seed germination on MS basal medium containing 4.8 g/L MS and 100 mg/L plant agar (Lee et al., 2009). Transgenic seedlings were grown in potting soil with photoperiodic lighting (LD 8:16) at a temperature of 22°C for 5 months. Pollen grains were then collected from the plants and stored at different temperatures: room temperature (RT), 4°C, -20°C, -70°C, or -196°C for 10 weeks.

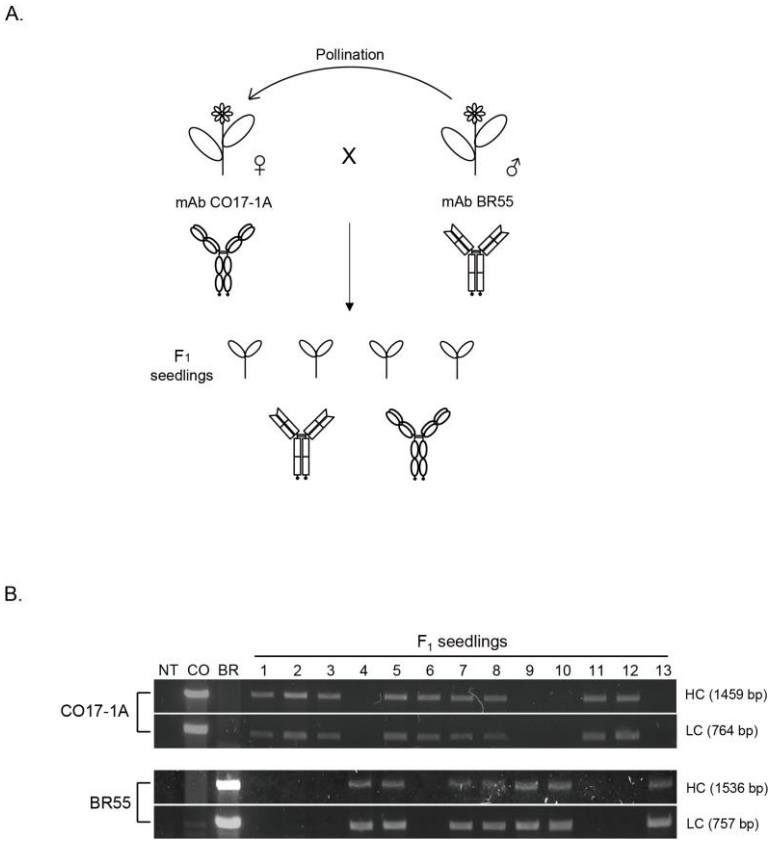

Fig 6. Schematic diagram of generating F_1 progenies from a cross between transgenic plants that express mAb^M BR55 and mAb^M CO17-1A to produce multiple mAbs in a single plant. A. The flowers of the CO17-1A transgenic plant were pollinated using pollen of the BR55 transgenic plant stored at -20°C. F_1 seeds were obtained from the cross between BR55 and CO17-1A transgenic plants and in vitro-germinated under Murashige and Skoog media containing kanamycin. B. PCR analysis of F_1 transgenic tobacco plant leaves. Fragments of the heavy chain (HC) and light chain (LC) were PCR amplified by using genomic DNA and electrophoretically separated on 1% agarose gel. NT: Non-transgenic plant, CO: Transgenic tobacco plant lines carrying the anti-colorectal cancer mAb CO17-1A HC and LC genes, BR: Transgenic tobacco plant lines carrying the anti-breast mAb BR55 HC and LC genes.

Pollen germination and pollen tube growth in sucrose buffer, with or without boric acid supplementation

Approximately 200 of the stored pollen samples were prepared on glass slides using 20 μL of either a solution of sucrose alone (20 μg/mL) or sucrose supplemented with boric acid (20 μg/mL each). The pollen samples stored at different temperatures (RT, 4°C, -20°C, -70°C, or -196°C) were sown on slide glasses. Slides were placed in Petri dishes lined with moist filter paper and incubated at 27.5°C for 2 h 30 min (Ahn et al., 2007; Modal et al., 2012). Pollen germination and pollen tube growth were observed under an optical microscope (Eclipse TS100; Nikon, Japan) at a magnification of ×100. To calculate the pollen germination rate (%), the number of germinated pollen grains was divided by the total number of observed pollen grains and multiplied by 100. To calculate the pollen germ tube growth rate (%) for each treatment group, the length of germinated pollen grains before storage was divided by the length of germinated pollens after storage, and multiplied by 100. The relative pollen germination rate was the percentage value ([germination rate of pollen stored for 10 weeks/germination rate of pollen before storage treatment] × 100). The relative pollen tube length growth rate was calculated in the following way: ([germ tube growth rate of pollen stored for 10 weeks/germ tube growth rate of pollen before storage treatment] × 100).

Statistical analyses

A Student's t-test was used to compare pollen germination and pollen germ tube growth between the boric acid treatments ($p < 0.05$), and ANOVA and Duncan's multiple range test ($p < 0.05$) in SPSS were used to compare them between the temperature treatment groups.

Generation of F_1 transgenic plants

The transgenic tobacco plant line BR55, which expresses the anti-breast mAb BR55, and tobacco plant CO17-1A, which expresses the anti-colorectal cancer mAb CO17-1A, were used in the present study (Kim et al., 2012; Ko et al., 2005). The

transgenic tobacco lines were grown in soil to induce blossoming, in order to obtain the flowers. The stamens of transgenic plant BR55 were collected prior to blossoming, and the pollen was collected. Collected pollen stored at -20°C was used to fertilize the stigma of the transgenic plant CO17-1A. The seeds obtained from the crossed plant were collected and sterilized with 10% Chlorox (Yuhan Crop, Inchun, Korea), 20% ethanol, and autoclaved water. The treated seeds were washed three times in distilled water and placed on MS media that consisted of 7 g/L of agar (Duchefa, Netherlands) and 4.8 g/L of MS (Sigma, St. Louis, MI, USA) containing kanamycin (100 mg/L). Seeds were maintained under photoperiodic lighting (LD 8:16) conditions at 22°C. As a negative control, seeds from non-transgenic plants were germinated in MS media without kanamycin.

Genomic DNA extraction and PCR analysis

To confirm whether the F_1 transgenic plants contained both the mAb BR55 and mAb CO17-1A *HC* and *LC* genes, genomic DNA was isolated from the plant leaves using a DNeasy kit (Qiagen, Germany), following the manufacturer's recommendations. PCR amplification was performed to confirm the presence of genes encoding the mAbBR55 *HC* and *LC* genes, by using the primers HCF (5′-TCTAGAATGCATCCAACGAGG-3′), HCR (5′-GATCCTTAA CCCGGAGTCCG-3′), LCF (5′-GCGGATCCATGCTACCG GCC-3′), and LCR (5′-CGCTGCAGTTAACATTCGTTCCTG-3′); for the mAb CO17-1A *HC* and *LC* genes, by using the primers HCF(5′-ATGGAATGGAGCAGAGTCTTT-3′), HCR (5′- ATCGATTTTACCCGGAGTCCG-3′), LCF (5′-ATGGGC ATCAAGATGGAATCA-3′), and LCR (5′-ACACTCATTC CTGTTGAAGCT-3′). PCR conditions for the *HC* and *LC* genes of the two mAbs were as follows: an initial denaturation at 94℃ for 2 min, followed by 30 amplification cycles consisting of a denaturation step at 94℃ for 20 s, annealing at 67℃ for 10 s, elongation at 72℃ for 50 s, and a final extension at 72°C for 5 min. A non-transgenic line was included as a negative control, and the genomic DNAs of individual transgenic BR55 plants, which expressed mAb BR55, and CO17-1A plants, which expressed mAb CO17-1A, were used as positive controls.

Conclusions

Our study revealed that BR55 pollen stored at -20°C is viable for pollination and fertilization, thus transferring both the *HC* and *LC* genes to the CO17-1A transgenic plant. We therefore conclude that boric acid positively affects pollen germination and germ tube growth in transgenic plants, indicating that it may play an essential role in both of these processes. We also found that storage temperature is an important factor that is involved in the pollen germination rate. Pollen stored at -20°C exhibited the highest rates of growth and germination after 10 weeks of storage, and exhibited high rates of viability for pollination and fertilization. The results of the present study provide the optimal storage temperature conditions for preserving the pollen of transgenic plants that carry useful recombinant genes, which can be used for the pollination of other plants. This information may be useful for the successful breeding of transgenic plants that express multiple recombinant protein genes.

Acknowledgments

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