Note

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A Comparison of GUS Activity after Liquid- and Air-Heat Shock Treatments in Transgenic *Nicotiana plumbaginifolia* Harboring the *Arabidopsis* HSP18.2 Promoter-GUS Chimeric Gene

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Abstract

We investigated the differences between liquid- and air-heat shock (HS) treatments performed on transgenic *Nicotiana plumbaginifolia* leaves harboring *Arabidopsis* HSP18.2 promoter-GUS chimeric gene. The optimum temperatures for heat-induced GUS activity in leaves were 42 ℃ for 2 h in liquid-HS treatments, and 45 ℃ for 2 h in air-HS treatments. Thus, we found a great difference between the GUS activity in leaves exposed to HS in air and liquid.

Plants produce many heat shock proteins (HSPs) which have a low molecular weight (LMW) (Vierling 1991). Takahashi *et al.* (1989, 1992) isolated an *Arabidopsis* LMW HSP18.2 promoter, and found that heat shock (HS) at 35 ℃ induced the maximum GUS activity in leaves of transgenic *Arabidopsis* harboring the *Arabidopsis* HSP 18.2 promoter-GUS chimeric gene. The HS treatment was given to the detached leaves submerged in a solution of MS salts (pH6.0), in a shaking water bath.

Spena *et al.* (1987) reported that HS at 40 ℃ induced high neomycin phosphotransferase (NPTII) activity in the separated roots, stems and leaves of transgenic tobacco harboring the *Drosophia* HSP70 promoter-NPT II chimeric gene. The HS treatment was then given to the leaves on MS agar medium in a petri dish in an incubater. The differences between the effects of HS given in air and liquid have not been compared in detail. In the present study, we performed short-term HS treatment in air (air-HS) and in liquid media (liquid-HS) using the leaves of transgenic *N. plumbaginifolia* harboring the *Arabidopsis* HSP18.2-GUS chimeric gene, and found a difference in the induced GUS activity.

Plant transformation

N. plumbaginifolia leaves were transformed using the leaf-disk procedure (Lloyed *et al.* 1986). Transformants harboring the HSP18.2-GUS fusion gene

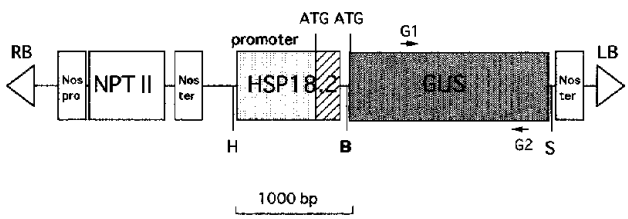

Fig. 1 Structure of the chimeric HSP18.2-GUS fusion gene.
HSP18.2 gene was excised from pTT119 (Takahashi *et al.* 1992) as the *Hin*dIII-*Bam*HI fragment and inserted into the binary vector plasmid pBI101.2. G-1 and G-2, are the approximate positions of the primers used for Genomic DNA analysis
; H *Hin*d III, B *Bam*H I, S *Sac* I.

(**Fig. 1**) were selected by callus formation and shoot regeneration on a Murashige and Skoog (MS) selection medium supplemented with 250mg l^{-1} kanamycin sulfate (KM). After rooting, they were transferred to soil-containing pots in growth chambers and grown under continuous fluorescent light (70 μ mol m^{-2} s^{-1}) at 26 ℃ for 5-7 months. We obtained 20 independently transformed lines.

The DNA purified by the Rapid Prep™ Micro Genomic DNA Isolation Kit (Pharmacia Biotech) from the leaves of transformed plants subjected to PCR to confirm the integration of the GUS gene in the genome. PCR was carried out using the 21 mer oligonucleotides (G-1, 5'-GGTGGGAAAGCGCGT

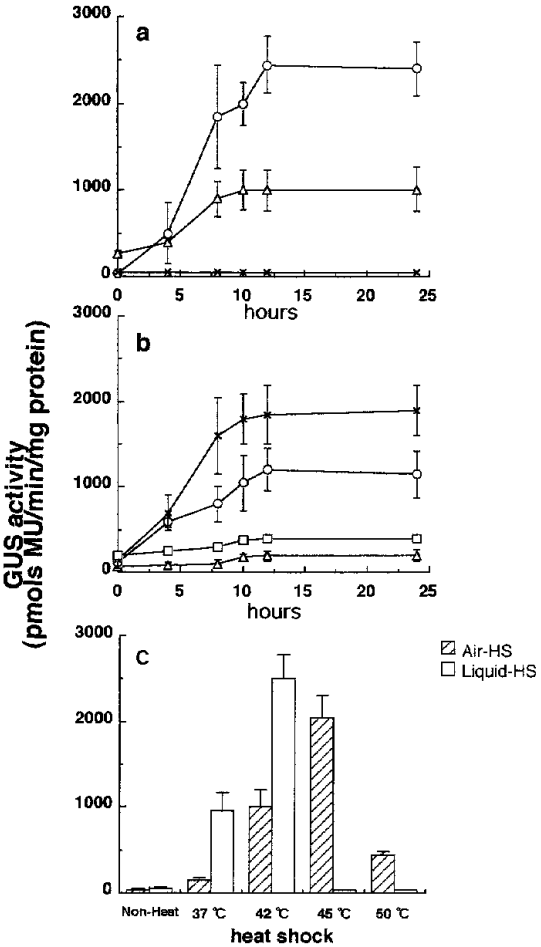

Fig. 2 Heat-induced GUS activity in transgenic *Nicotiana plumbaginifolia* leaves after air- and liquid-heat shock treatments. Leaves (length 5-8 cm; line HG-7) which showed high GUS activity were incubated at various temperatures for 2 hr. Progeny from HG-7 line segregated at a ratio close to 3:1 for GUS activity. (a) GUS activity measured at various hours after liquid-HS (37 ℃ (△), 42 ℃ (○) and 45 ℃ for 2 h(x)). (b) GUS activity measured at various hours (normal temperatures) after air-HS (37 ℃ (△), 42 ℃ (○), 45 ℃ (x)and 50 ℃ for 2 h(□). (c) The maximum GUS activity after air- and liquid-HS (non-heat, 37℃, 42℃, 45℃ and 50 ℃ for 2 h). Hatched bar (left), air-HS treatment; Open bar (right), liquid-HS treatment. Bars represent the standard errors of the means of three lines. GUS activity was analyzed by the method of Jefferson *et al.* (1987), and shown in units of pmol 4-methylumbelliferone (MU) formation per milligram of protein per minute.

TACAAG-3' and G-2, 5'-GTTTACGCGTTGCTT CCGCCA-3') (Hamill *et al.* 1991) in a 25μl reaction solution containing 1 ng DNA, 5 pmol of each primer, 0.2μl Taq Extender™ PCR additive (Stratgene), 0.3μl Taq Start™ Antibody (Clontech) and 0.2 mM dNTP, with 35 cycles of 30 s at 94 ℃, 2 min at 58 ℃, and 3 min at 72 ℃. The amplified DNA was electrophoresed in 1 % agarose gel. The expected unique band (1.2 Kb) of PCR products was then detected in all transgenic plants, while it was not detected in wild-type plants (data not shown).

GUS activity after liquid- and air-HS treatments

HS treatments were given by two methods, 1) liquid-HS, leaves were incubated in a solution (10 ml) of MS salts (pH 5.8) in a shaking water bath, and 2) air-HS, leaves kept on an agar medium containing MS salts were incubated in a growth chamber. Leaves after liquid- and air-HS treatments were kept on an agar medium containing MS salts for various hours at normal temperatures (20-22 ℃). The water bath and growth chamber temperatures were controlled at the accuracy in the order of 0.1 ℃.

The GUS activity in the leaves increased gradually at normal temperatures after liquid-HS treatment at (37 ℃ or 42 ℃ for 2 h), and reached the maximum level at the 10-12th h (**Fig. 2a**). The activity did not significantly change thereafter until the 24th h. The GUS activity after air-HS treatment also increased like that after liquid-HS treatment (**Fig. 2b**). However, the activity after HS at 37 ℃ and 42 ℃ in air was lower than that after the same HS in liquid. The activity after air-HS at 45 ℃ was higher than that at 42 ℃ or 37 ℃ and even HS at 50 ℃ in air induced some activity, although the activity after liquid-HS at 45 ℃ was negligible (**Fig. 2a and b**). Heat-induced GUS activities in transgenic lines HG-5 and HG-13 also showed similar patterns to that of transgenic line HG-7. Thus, we found a great difference between the GUS activity in leaves exposed to HS in air and liquid. However, the temperatures of the surface of leaves analyzed by an infrared thermometer (Horiba, IT-340) in the growth chamber was 3-4 ℃ lower than the temperatures indicated by thermometer during air-HS. Accordingly, the result can be correlated to differences of the GUS activity in leaves exposed in air- and liquid-HS. However, for example, we can't explain the GUS activity induced even after air-HS at 50 ℃ (temperatures of leaves-surface, 46-47 ℃) although over 45 ℃ in liquid-HS induced little. Further, we will need to investigate the differences.

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Note

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Production of Non-organogenic and Loosely Attached Callus in Leaf Disk Cultures of Haploid *Nicotiana plumbaginifolia* by ^{14}N Ion Beam Irradiation

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Abstract

The leaf disks of Nicotiana plumbaginifolia were irradiated by an ^{14}N heavy-ion beam and then were cultured in shoot-inducing medium in order to produce mutants for elucidating the functions of the cell wall in plant morphogenesis. Non-organogenic and loosely attached callus, which had the same morphological features as non-embryogenic callus of carrot was formed on 11.8% of the haploid leaf disks irradiated by ^{14}N heavy-ion beam (5 Gy) at high frequency. The cells stimulated by the heavy-ion beam loosely attached to each other resulting in a random morphology of cell clusters, whereas the non-irradiated cells formed a tight callus with multiple shoots.

During long-term culture of carrot embryogenic callus in suspension, the callus gradually begins to form small clusters of cells and loses embryogenic competence, becoming non-embryogenic callus (Reinert, *et al.* 1970; Satoh, *et al.* 1986; Zimmerman, 1993). In culture systems derived from other plants, such as tobacco and rice, loss of morphogenic competence (the capacity for embryogenesis and formation of adventitious buds) is generally observed with an accompanying reduction in the size of cell clusters as a result of the loosening of intercellular attachments (Satoh, 1998).

Differences between embryogenic callus and non-embryogenic callus were also found in the morphological, biochemical and histochemical features of the cell walls (Kikuchi, *et al.* 1995; Kikuchi, *et al.* 1996a, b; Iwai, *et al.* 1999).

Although the somaclonal variations involved in cell wall synthesis and function may result in weak intercellular attachment in non-embryogenic callus, it is hard to further analyze those mutants because the variation sometimes occurs in chromosomal level. Consequently, in this study, we tried to produce the mutants involved in intercellular attachment for elucidating the function of cell wall components using the haploid plants of *Nicotiana plumbaginifolia*. Haploid plants are desirable for making mutants in tissue culture because recessive mutations appear directly in the phenotype in such plant. Moreover, *N. plumbaginifolia* has a minimum set of genomes in *Nicotiana* and established culture systems (Negruitiu, *et al.* 1983; Presting and Helgeson, 1994).

Because the heavy-ion beam irradiation possesses a much higher linear energy transfer (LET) to the target than the ionizing radiation (Tanaka, *et al.* 1997)., the former requires a considerably lower dose for the mutation; moreover, secondary deleterious effects can be minimized (Kiefer, 1985; Suzuki, *et al.* 1996; Tanaka, *et al.* 1997; Abe, *et al.* 1997; Abe, *et al.* 1998).

All samples were irradiated with a 135 MeV/u ^{14}N ion beam using the RIKEN Ring Cyclotron. Linear energy transfer (LET) of the ^{14}N ion corresponded to 34 keV/μm. Leaves of haploid and diploid *Nicotiana plumbaginifolia*, which had been gifted from Institut National de la Recherche Agronomique (INRA) Centre de Versailles in France, placed in 60 x 15 mm plastic petri dishes (Falcon; Becton Dickinson & Company, New Jersey, U.S.A.) aseptically were exposed to the ^{14}N ion beam for specific periods of time, corresponding to doses ranging from 1.25 to 40 Gy, and then the leaves (including veins) were immediately cut into pieces (approximately 6 x 6 mm). The leaf disks were transferred to a Murashige and Skoog's agar medium containing 1 mg/*l* benzyl adenine for shoot induction; then the plates were incubated under continuous light conditions at 28 ℃. The effects of irradiation were evaluated after three months of culture. The normal callus formed hard cell-clusters with multiple shoots, but paste-like callus with

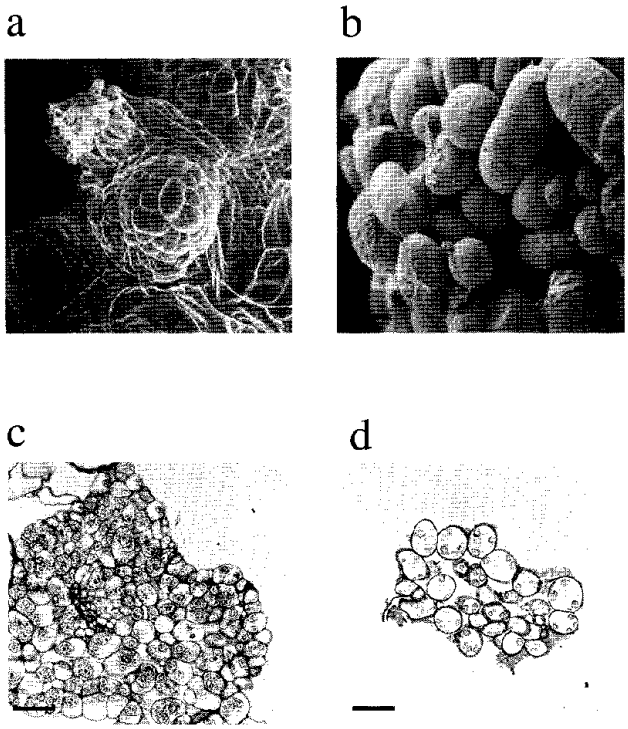

Fig. 1 Morphological features of the callus formed on the leaf disks. Normal callus (a and c) and non-organogenic and loosely attached callus formed on the leaf disks irradiated by ^{14}N heavy-ion beam (b and d) were observed by scanning electron microscope (a and b) and light microscope (c and d). The bars represent 50 μm.

weak intercellular attachments was found in the irradiated culture. We confirmed the characteristics of the paste-like loosely attached callus by touching it with tweezers. The survival rate was defined as the percentage of leaf disks that avoided entire browning. The frequency of cell proliferation was defined as the percentage of leaf disks on which callus and/or adventitious shoots formed.

Light microscopic and scanning electron microscopic observations were carried out as described previously (Sakuta, *et al.* 1998; Iwai, *et al.* 1999).

Non-irradiated leaf disks began to form the callus with multiple shoots and adventitious shoot after about ten days of culture. However, browning of some leaf disks was observed in those that had received irradiation. The frequency of cell proliferation decreased in parallel with increasing dose in both haploid and diploid leaf disks, and the frequency of cell proliferation in diploid was higher than that in haploid (data not shown). At the highest dose of irradiation (40 Gy), almost complete inhibition of cell proliferation was observed in the leaf disks of both haploid and diploid plants. A decrease of survival rate (up to 80%) was observed at 40 Gy in the haploid plants; there was no change in the diploid plants (data not shown).

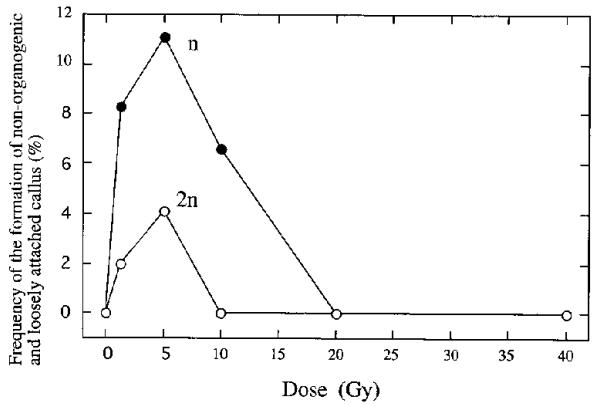

Fig. 2 Effects of the dose of ^{14}N-ion beam on the production of non-organogenic and loosely attached callus. Results are expressed as percentage of the leaf disks on which the non-organogenic and loosely attached callus was formed.

In the culture with the irradiation, the formation of non-organogenic and loosely attached callus was observed. The morphological characteristics of callus were examined by scanning electron microscopy (**Fig. 1 a, b**) and light microscopy (**Fig. 1 c, d**). In normal callus, multiple shoots were formed on the surface of the callus (**Fig. 1 a**) and the cells formed tightly attached large cell-clusters (**Fig. 1 c**). In contrast, the cells of non-organogenic callus formed on the irradiated leaf disks were a little larger and loosely attached to each other (**Fig. 1 d**), resulting in a random morphology of cell clusters (**Fig. 1 b**). Larger vacuoles and a lower number of chloroplasts were also observed in the non-organogenic and loosely attached callus (**Fig. 1 d**).

The frequency of formation of non-organogenic and loosely attached callus in the culture of haploid leaf disks was much higher than that of the diploid disks (**Fig. 2**). The best frequency was obtained at 5 Gy of irradiation in the cultures of both haploid (11.8%) and diploid (4.1%) leaf disks.

Long term culture for several months has been known to occasionally stimulate spontaneous somaclonal variations under culture conditions with a high concentration of auxin as seen in carrot culture system (Larkin and Scowcroft, 1981; Liscum, and Hangarter, 1991; Satoh, *et al.* 1998). In our culture systems, the auxin was not added in the medium at all, and only cytokinin was added as a growth substance. Therefore, the spontaneous somaclonal variations should have been minimized in our culture system. In our system, non-organogenic and loosely attached callus were stimulated at a high rate (ca. 10% of leaf disks) by irradiation with a heavy-ion beam, and their frequency in the case of haploids were higher than in case of diploids. These

results suggest that non-organogenic and loosely attached callus appeared as a result of mutation produced by the heavy-ion beam irradiation.

These results show that ^{14}N ion beam irradiation is a powerful tool for obtaining mutants at high frequency through the use of the haploid plants. The non-organogenic and loosely attached callus obtained in this experiment provided valuable materials for elucidating the functions and the synthesis of cell wall polysaccharides in further studies of the mechanisms of intercellular attachments in higher plants.

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