I.1 Somatic Hybridization Between *Hordeum vulgare* L. (Barley) and *Daucus carota* L. (Carrot)

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1 Introduction

The aim of plant breeding is to construct new genotypes by the introduction and manipulation of genetic variations. The production of somatic hybrid plants by protoplast fusion is a potentially useful method for the combination of genetic materials. Protoplast fusion can sometimes lead to the production of new genetic variants as a consequence of the recombination of nuclear and/or of cytoplasmic genomes. Many intra- and interspecific and several intergeneric somatic hybrid plants have been reported (Melchers et al. 1978; Aviv et al. 1980; Gupta et al. 1982, 1984; Menczel et al. 1983; Negrutiu et al. 1986; Pental et al. 1986; Toriyama et al. 1987; Gleba et al. 1988; Kameya et al. 1989; Toki et al. 1990; Kostenyuk et al. 1991; Perl et al. 1991; Babiychuk et al. 1992). Recently, asymmetric hybrids between remotely related species, for example interfamilial hybrid plants have been remotely exploiting various systems for the selection of hybrids (Somers et al. 1986; Dudits et al. 1987; Kisaka and Kameya 1994; Kisaka et al. 1994).

Intergeneric fusion of *Solanum* and *Lycopersicon* species as a means of introducing tolerance to certain environmental stresses has been reported (Melchers et al. 1978; O'Connell and Hanson 1986). Cold tolerance of plants that were somatic hybrids of potato and tomato was reported by Smillie et al. (1979). Recently, transgenic tobacco plants carrying a bacterial gene for mannitol-1-phosphate dehydrogenase were shown to have enhanced ability to tolerate high salinity as a result of the accumulation of mannitol (Tarczynski et al. 1993). Deping et al. (1996) also produced transgenic rice that introduced a late embryogenesis abundant (LEA) protein gene, the *HVA1* gene, from *H. vulgare*. The transgenic rice plants were also shown to have enhanced ability to tolerant to water deficit and high salinity. Furthermore, transgenic rice plants carrying the gene for betaine aldehyde dehydrogenase or the *codA* gene for choline oxidase were shown to have enhanced ability to tolerate high salinity (Nakamura et al. 1997; Sakamoto et al. 1998).

Barley (*Hordeum vulgare* L.) is a crop plant that tolerates low temperature and salinity. The possibility is examined that these characteristics of barley might be transferable to somatic hybrids of barley and carrot (*Daucus carota*

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Biotechnology in Agriculture and Forestry, Vol. 49
Nagata/Bajaj (Eds.) Somatic Hybridization in Crop Improvement II

L.), utilizing the low-temperature tolerance of barley for selection of hybrids, and that calli induced from the somatic hybrids to determine whether the cold and salt tolerance of *H. vulgare* had been transferred to it by the original protoplast fusion.

2 Protoplast Fusion and Culture of Fused Cells

When cells from 6-month-old suspension cultures of cells isolated from *D. carota* were plated on medium D and incubated at 4 °C for various periods and the calli then transferred to 25 °C, the number of the regenerated calli decreased with increasing duration of the low-temperature treatment (Fig. 1). On the basis of the result, the low-temperature treatment for selection of hybrid calli consisted of incubation at 4 °C for 5 weeks after incubation for 1 month at 25 °C of fused cells.

Protoplasts of *D. carota* isolated from 6-month-old suspension culture, and those of *H. vulgare* isolated from young leaves were fused by electrofusion, and fused cells were cultured according to the scheme outlined for selection of hybrids in Fig. 2. After culture for 1 month at 25 °C, the fused cells were transferred to medium D and then incubated at low temperature (4 °C) for 5 weeks in darkness (Fig. 3a) The resultant calli were transferred to continuous light (4 Wm^{-2}) at 25 °C. After visible colonies had developed to about 1–2 mm in diameter, about 2700 colonies were transferred to fresh medium D. Three shoots were regenerated (Fig. 3b) and these were transferred to medium E. The three regenerated plants were potted in soil and designated nos. 1, 2, and 3 (Fig. 3c).

Protoplasts of *H. vulgare* that were isolated from young leaves failed to divide. Protoplasts of *D. carota* that were isolated from 6-month-old suspension cultures proliferated and formed colonies. However, about 1400 colonies

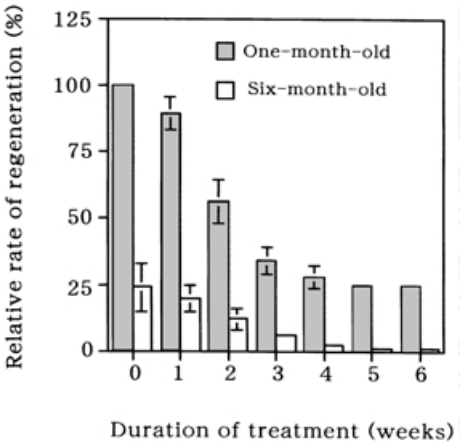

Fig. 1. Effects of low-temperature treatment on cell regeneration. One-month-old and 6-month-old calli of *D. carota* were cultured at 4 °C for various periods, and then transferred to regeneration medium and cultured at 25 °C for 6 weeks. The regeneration rate in control cells (1 month old) without low-temperature treatment was taken as 100%

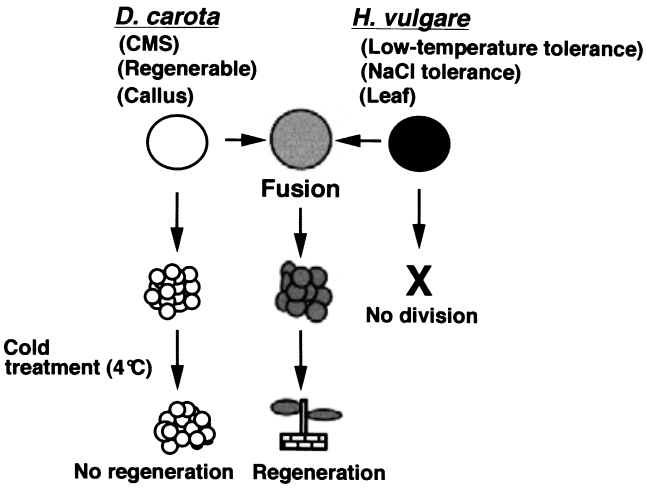

Fig. 2. Scheme for selection of hybrids between *H. vulgare* and *D. carota*

that had been incubated at 4 °C for 5 weeks failed to regenerate any shoots. Furthermore, no plants were obtained from protoplasts of either *H. vulgare* or *D. carota* that were cultured under the same conditions without fusion treatment.

3 Analysis of the Three Regenerated Plants

The somatic hybrid plants closely resembled *D. carota* in morphology (Fig. 3d, e). No. 1 hybrid had variegated green and white leaves and flowers which developed without vernalization (Fig. 3g, h, i). The morphology of roots of the somatic hybrids was similar to that of roots of *D. carota* (Fig. 3f). The flowers exhibited male sterility, as did those of the parent strain of *D. carota*.

Callus cultures induced from leaf segments of the regenerated plants and their parents were analyzed at the cytological and molecular levels. Cytological analysis revealed that the chromosome number of the regenerated plants was about 24 (Table 1), namely, significantly lower than the sum of the chromosome numbers (32) of the parents. Genomic DNA was analyzed by Southern hybridization with a nonradioactively labeled DNA fragment of the *rgp1* gene. The regenerated plants generated both a band specific for *D. carota* (4.4 kbp) and a band specific for *H. vulgare* (3.6 kbp) (Fig. 4). Chloroplast (ct) and mitochondrial (mt) DNAs were also analyzed by Southern hybridization with fragments of ctDNA and mtDNA (Table 1). The results of analysis of ctDNA with a non-radioactively labeled fragment of rice ctDNA of *Bam*HI-8 as probe indicated that the regenerated plants yielded both bands specific for *D. carota* (4.2 and 2.2 kbp) and a band specific for *H. vulgare* (9.0 kbp)

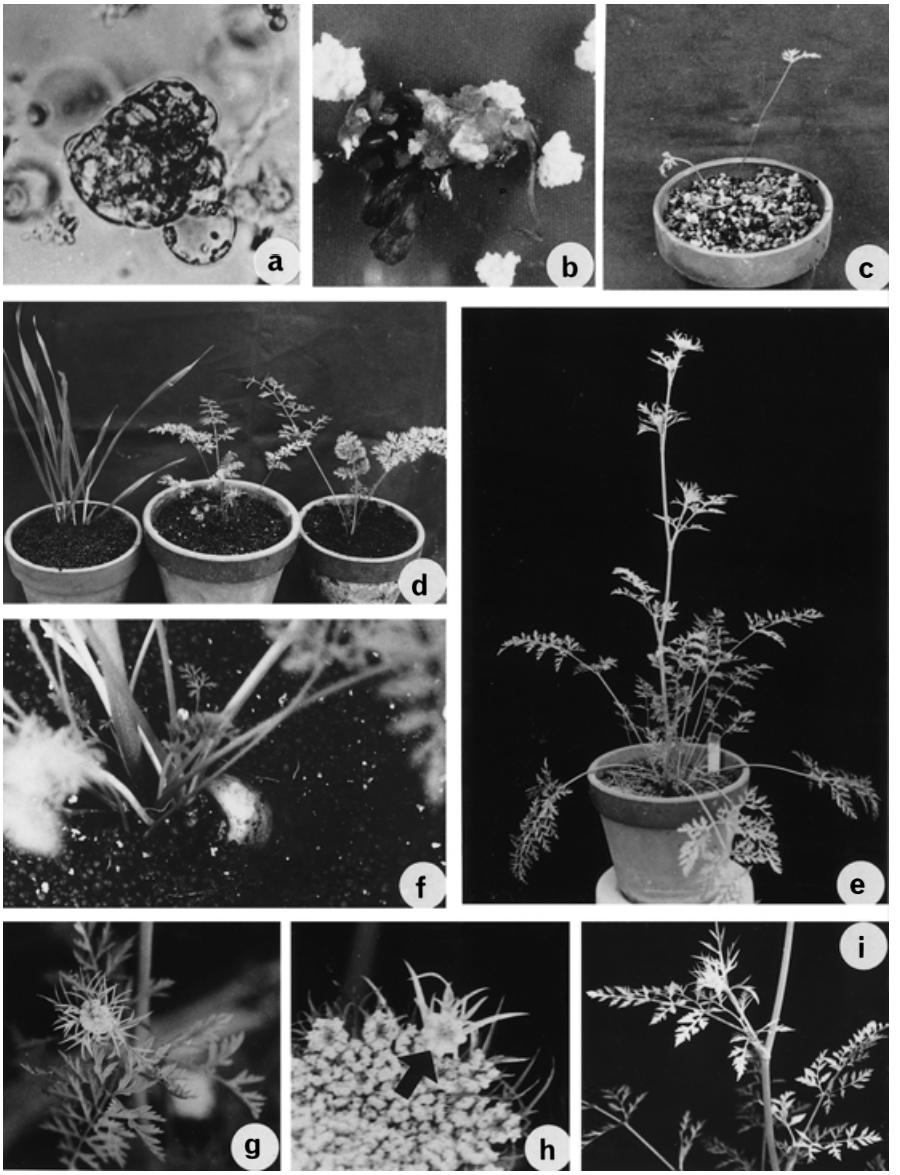

Fig. 3. **a** Multicellular after 2 weeks of culture. **b** Regeneration of shoots from selected callus. **c** Regenerated plant potted in soil. **d** *Left to right* Plants of *H. vulgare*, a regenerant, and *D. carota*. **e** Plant of somatic hybrid (no. 1). **f** Root of somatic hybrid (no. 1). **g–i** Green and white flowers (**g, h**) and leaves (**i**); *arrow* (**h**) indicates a white flower

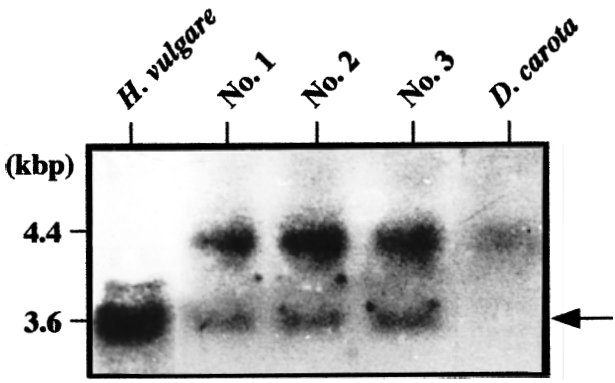

Fig. 4. Results of Southern hybridization analysis of genomic DNA. Total DNA was digested with *Hin*dIII, and a fragment of the *rgp1* gene was used as probe. *Arrow* indicates a band specific for *H. vulgare*

Table 1. Chromosome number and cytoplasmic genotype

Cell line	Chromosome no.	Fragments of mtDNA					Fragments of ctDNA				
		atp6	*atp9*	*cob*	18s rRNA	26s rRNA	pSB3	pSB8	pSB10	pSB13	pSB16
No. 1	24.4 (± 1.4)[a]	D + N	DC	DC	DC	DC	H + N	D + H	DC	DC	DC
No. 2	24.9 (± 2.6)	DC	DC	DC	DC	DC	H + N	D + H	DC	DC	DC
No. 3	24.2 (± 1.8)	DC	DC	DC	DC	DC	H + N	D + H	DC	DC	DC

[a] Standard error ($n > 10$). D + N, *D. carota* band plus novel band; H + N, *H. vulgare* band plus novel band; D + H, *D. carota* band plus *H. vulgare* band; DC, *D. carota* type.

(Fig. 5a). The regenerated plants also yielded a band specific for *H. vulgare* (4.4 kbp) and a unique band (8.6 kbp) when the *Bam*HI-3 fragment of rice ctDNA was used as the probe (Fig. 5b). In the analysis of mtDNA, one of the regenerated plants (no. 1) yielded a novel band (9.0 kbp) that was not detected in the analysis of either parent when a fragment of *atp6* was used as the probe (Fig. 6). These results indicated that the regenerated plants were somatic hybrids of *H. vulgare* and *D. carota*.

4 Characterization of Somatic Hybrids

Cells in suspension cultures induced from three somatic hybrid plants and their parents were incubated at 4 °C for various hours after culture for 1 week at 25 °C. From the results of measurement of fresh weight of cold-treatment cells, the growth of *H. vulgare* and no. 2 was more than that of *D. carota* and other somatic hybrids (nos. 1 and 3) in the cell levels (Fig. 7a). TTC-reduction

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Somatic Hybridization in Crop Improvement II
Nagata, T.; Bajaj, Y.P.S. (Eds.)
2001, XXI, 378 p. 146 illus., 5 illus. in color., Hardcover
ISBN: 978-3-540-41112-3