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Original Paper

Evaluation of the potential for somatic embryogenesis in sugar beet (*Beta vulgaris* L.) breeding lines and improvement of regeneration efficiency

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Abstract A wide range of genotypes suitable for a plant regeneration system is needed when utilizing genetic transformation techniques to develop new crop cultivars. We examined the regeneration efficiencies for somatic embryogenesis in 61 genetically diverse sugar beet (*Beta vulgaris* L.) breeding lines developed in Japan using a previously reported procedure. Frequencies of embryogenic callus formation from seedling leaf and petiole explants ranged from 0 to 89% and those of somatic embryo formation from the calli ranged from 0 to 99%. There was no clear correlation between the two frequencies, suggesting that different genetic backgrounds are involved in the two formation mechanisms. To evaluate each breeding line's capability for somatic embryogenesis, we proposed a somatic embryogenesis capability index based on four parameters. Based on the values of this index, only 21 breeding lines were suitable materials for regeneration via somatic embryogenesis. These breeding lines include four lines with moderate or better resistance to three major sugar beet diseases in Japan and one line with strong resistance to these diseases, and these lines will be useful materials with superior characteristics for developing transgenic breeding lines. To increase the regeneration efficiencies in the other 40 breeding lines, we examined other types of explants from seedlings and the effects of various plant growth regulators. We found that cotyledon explants generally showed improved callus formation and that using thidiazuron instead of N^6-benzylaminopurine improved somatic embryo formation in some lines.

Key words: Cotyledon explant, embryogenic callus, somatic embryo, sugar beet (*Beta vulgaris* L.), thidiazuron (TDZ).

Sugar beet (*Beta vulgaris* L.) is a staple crop for sugar production in the temperate zones of the world. In the Hokkaido region, the only sugar beet cultivation area in Japan, commercial sugar beet cultivars with high yields and some disease resistance have been developed by the Hokkaido Agricultural Research Center, National Agriculture and Food Research Organization (NARO/HARC) or introduced from abroad by Japanese sugar production companies. Many of these cultivars have been cultivated in the Hokkaido region, but farmers have requested materials with more desirable agricultural characteristics to promote stable production with lower costs and lower labor requirements. However, some of these characteristics cannot be incorporated into new cultivars by means of cross-breeding techniques because there are no genetic resources of sugar beet that possess these characteristics. For example, freezing tolerance to protect against a late frost during the early seedling stage is needed when using a direct-sowing cultivation method that greatly reduces labor requirements compared with a transplanting cultivation method, but sugar beet genetic resources that possess this characteristic have not yet been identified (Matsuhira et al. 2012b). Lack of such genetic resources could be compensated by transferring the genes responsible for the freezing tolerance from another organism habituating low temperature.

Abbreviations: BAP, N^6-benzylaminopurine; CPPU, forchlorfenuron (N-(2-chloro-4-pyridyl)-N′-phenylurea); ICA, index of the callus amount; NSE, number of somatic embryos per callus; PEC, percentage of embryogenic callus formation; PSE, percentage of somatic embryo formation; TDZ, thidiazuron.

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Various genetic transformation techniques can be utilized to introduce genes that confer desirable characteristics and develop new cultivars when it is difficult to introduce such genes by means of conventional cross-breeding techniques. The development of an efficient plant regeneration system that can be applied to a wide range of sugar beet genotypes is a prerequisite for utilizing such genetic transformation techniques. Many researchers have reported procedures for the induction of adventitious shoot regeneration and somatic embryogenesis in sugar beet cultivars and breeding lines (Abe et al. 1991; Dovzhenko and Koop 2003; Freytag et al. 1988; Jacq et al. 1992; Krens and Jamar 1989; Roussy et al. 1996; Saunders and Daub 1984; Saunders and Shin 1986; Saunders and Tsai 1999; Tétu et al. 1987; Tsai and Saunders 1995; Zhang et al. 2001, 2004, 2008). Regeneration via somatic embryogenesis is regarded as a promising pathway for *Agrobacterium*-mediated transformation to avoid the production of genetic chimeras, because a somatic embryo is assumed to be derived from a single callus cell (Zhang et al. 2008).

An efficient somatic embryo induction system has been reported for some of the sugar beet breeding lines developed by NARO/HARC (Tamagake 1999). In this method, embryogenic callus is induced from the hypocotyls or the leaves and petioles of seedlings. Among the breeding lines that were examined, significant variations were observed in the frequencies of embryogenic callus formation and somatic embryo formation, and the breeding line NK-219 mm-O exhibited the highest frequencies of formation of both materials. Subsequently, an *Agrobacterium*-mediated transformation system via somatic embryogenesis was developed for breeding line NK-219 mm-O (H. Tamagake, unpublished data). However, as this breeding line has some drawbacks, such as disease susceptibility, immediate use of transgenic NK-219 mm-O for breeding purposes will be difficult, although it remains useful for studies of gene expression (Matsuhira et al. 2012a). It is therefore necessary to find other breeding lines that exhibit regeneration efficiencies comparable to that of NK-219 mm-O, but with superior agricultural characteristics, and to increase the regeneration efficiencies of any breeding lines for which somatic embryogenesis is currently difficult.

In this study, we investigated 61 sugar beet breeding lines to assess the efficiencies of callus formation and somatic embryo formation, and we evaluated each line's potential for somatic embryogenesis by developing a suitable evaluation index. To increase the efficiencies of the regeneration system for breeding lines with lower values of this index, we examined explants from different seedling organs and the application of various plant growth regulators in the culture medium.

Materials and methods

Basal medium

The basal medium used in this study was composed of modified MS medium (Murashige and Skoog 1962), in which the amount of NH_4NO_3 was adjusted to 825 mg l^{-1}, and the medium was supplemented with 30 g l^{-1} sucrose, 0.25 g l^{-1} 2-morpholinoethanesulfonic acid, and 2.5 g l^{-1} Gelrite (Wako, Tokyo, Japan). This medium was autoclaved at 121°C for 10 min, then was further supplemented with plant growth regulators (described later in the Methods) and adjusted to pH 5.8.

Plant materials

The 61 sugar beet breeding lines used in this study were monogerm, O-type (a maintainer of cytoplasmic male sterility) inbred lines that had been developed by NARO/HARC (Table 1). Seeds of the breeding lines were provided by the NARO/HARC Sugar Beet Breeding Group.

Seed sterilization and sowing

After soaking the seeds in concentrated H_2SO_4 for 30 to 40 min to remove the pericarps, the seeds were washed overnight with running water. The seeds were then surface-sterilized by successive immersion in 70% (v/v) ethanol for 1 min and 0.5% (v/v) sodium hypochlorite for 15 min, followed by three washes with sterile distilled water. The sterilized seeds were cultured for germination in 9-cm disposable plastic Petri dishes containing 25 ml of the basal medium under fluorescent light (30 to 40 μmol m^{-2} s^{-1}) at 25°C, with a 16 h light/8 h dark photoperiod.

Embryogenic callus induction

Germinated seedlings from the cultured seeds were transferred into 200-ml culture bottles containing 40 ml of the basal medium, but with 8 g l^{-1} agar instead of 2.5 g l^{-1} Gelrite, and were grown under the same conditions described previously. In each breeding line, 30 germinated seedlings free from bacterial or fungal contamination were grown. When the 3rd true leaves of the seedlings were about 3 cm long (about 3 weeks after transfer), three true leaves and petioles were excised from each seedling and cut into pieces about 7 mm long. They were then cultured on the basal medium supplemented with 1.0 mg l^{-1} N^6-benzylaminopurine (BAP; callus-induction medium) in 9-cm disposable plastic Petri dishes in the dark at 25°C. After about 2 months, the number of explants that had formed an embryogenic callus in each Petri dish was counted. We used this to calculate the percentage of embryogenic callus formation (PEC) from each seedling and its average for 30 Petri dishes in each breeding line. We also examined the callus amount in each explant that formed an embryogenic callus. The index of the callus amount (ICA) was defined based on the diameter of the callus mass: 0.2, for $<$1 mm; 1, 1 to $<$5 mm; 2, 5 to $<$10 mm; 3, 10 to $<$15 mm; 4, $\geq$15 mm.

Table 1. The percentage of embryogenic callus formation (PEC) and the index of the callus amount (ICA) for the cultured leaf and petiole explants of 61 sugar beet breeding lines.

Breeding line	PEC (%)	ICA[a]	Breeding line	PEC (%)	ICA[a]
NK-185BRmm-O	0±0	—	NK-182mm-O	37.2±4.4	1.2±0.1
NK-194mm-O	0±0	—	NK-328mm-O	39.4±4.2	1.4±0.1
NK-208BRmm-O	0±0	—	NK-294mm-O	40.6±4.8	1.4±0.1
NK-237BRmm-O	0±0	—	NK-242mm-O	43.9±4.3	1.2±0.1
NK-239BRmm-O	0±0	—	NK-326mm-O	43.9±5.5	1.3±0.1
NK-246BRmm-O	0±0	—	NK-235BRmm-O	44.4±6.9	1.3±0.1
NK-248BRmm-O	0±0	—	NK-184mm-O	45.6±5.9	1.3±0.1
NK-300mm-O	0±0	—	NK-180BRmm-O	46.7±6.6	1.4±0.2
NK-245BRmm-O	0.6±0.5	1.0±0	NK-197mm-O	46.7±4.4	1.1±0.1
NK-229BRmm-O	1.1±0.8	0.2±0	NK-190mm-O	50.0±5.9	1.2±0.1
NK-290BRmm-O	1.1±0.8	1.0±0	NK-181BRmm-O	50.6±7.2	1.3±0.1
NK-329mm-O	1.1±0.8	0.6±0	NK-310mm-O	50.6±6.0	1.8±0.2
NK-214mm-O	6.1±2.3	1.4±0.1	NK-203mm-O	51.1±4.6	1.2±0.1
NK-206mm-O	8.3±3.0	0.9±0.1	NK-213BRmm-O	53.3±5.8	1.6±0.2
NK-195BRmm-O	10.0±3.7	1.4±0.1	NK-226BRmm-O	53.3±5.1	1.4±0.1
NK-301mm-O	11.1±3.0	1.1±0.1	NK-295BRmm-O	58.3±5.8	1.2±0.1
NK-324mm-O	11.1±2.6	1.1±0.1	NK-299mm-O	58.9±6.9	1.5±0.1
NK-254BRmm-O	12.8±3.2	1.2±0.1	NK-193mm-O	59.4±6.1	1.4±0.1
NK-311mm-O	12.8±4.8	1.0±0.1	NK-256BRmm-O	60.0±5.6	1.4±0.1
NK-244BRmm-O	15.6±4.7	1.6±0.1	NK-230BRmm-O	61.7±6.7	1.3±0.1
NK-323mm-O	16.7±2.8	1.1±0.1	NK-307mm-O	65.6±6.7	1.7±0.2
NK-233mm-O	20.6±2.9	1.0±0.1	NK-204mm-O	67.8±6.6	1.8±0.1
NK-227BRmm-O	27.8±6.2	1.4±0.1	NK-215mm-O	68.3±7.0	1.1±0.1
NK-221mm-O	28.3±6.8	1.5±0.1	NK-291BRmm-O	69.4±4.2	1.5±0.1
NK-191mm-O	29.4±4.6	1.2±0.1	NK-327mm-O	72.8±6.0	1.6±0.1
NK-196mm-O	29.4±4.5	1.2±0.1	NK-207mm-O	80.6±4.6	1.8±0.1
NK-183BRmm-O	31.7±4.2	1.2±0.1	NK-220BRmm-O	81.1±4.4	1.1±0.1
NK-313mm-O	32.2±5.9	1.0±0.1	NK-205mm-O	81.7±4.7	1.3±0.1
NK-234BRmm-O	33.3±4.4	1.3±0.1	NK-236BRmm-O	83.9±4.0	1.2±0.1
NK-306mm-O	33.3±5.3	1.0±0.1	NK-219mm-O	88.9±5.5	2.3±0.2
NK-255BRmm-O	36.7±5.4	1.4±0.1			

[a] Values of ICA are defined based on the diameter of the callus mass: 0.2, <1 mm; 1, 1 to <5 mm; 2, 5 to <10 mm; 3, 10 to <15 mm; 4, ≥15 mm. Values are the mean±SE.

Somatic embryo induction

Callus pieces (each about 3 mm in diameter), or the entire callus mass if it was smaller than 3 mm, were taken from each embryogenic callus that formed and were subcultured on the basal medium supplemented with 1.0 mg l^{-1} BAP, 1.0 mg l^{-1} abscisic acid, and 1.0 mg l^{-1} 2,3,5-triiodobenzoic acid (somatic embryo-induction medium) and incubated under fluorescent light (30 to 40 μmol m^{-2} s^{-1}) at 25°C, with a 16 h light/8 h dark photoperiod (n=1 to 160 calli per breeding line). After about 1 month, we determined whether somatic embryos had formed from each callus, and calculated the percentage of somatic embryo formation (PSE) in each breeding line. We also counted the number of somatic embryos (NSE) that formed on each callus, and calculated the average number for each breeding line. For subsequent growth, the somatic embryos were subcultured on the basal medium supplemented with 0.25 mg l^{-1} BAP and incubated under fluorescent light (30 to 40 μmol m^{-2} s^{-1}) at 25°C, with a 16 h light/8 h dark photoperiod.

Improvement of the efficiencies of embryogenic callus formation

Cotyledons of the seedlings in the breeding lines with a relatively low frequency (<30% PEC) of embryogenic callus formation were cut into two pieces, each 3 to 7 mm long (n=3 seedlings per breeding line), when the true leaves were 1 to 3 mm long and were cultured on the callus-induction medium (four pieces per seedling) in the dark at 25°C. At the same time, hypocotyls of the seedlings were cut into two pieces, each 3 to 5 mm long, and were cultured on the callus-induction medium under the same conditions. After about 2 months, we calculated the percentage of the explants that formed an embryogenic callus and used ICA to describe the size of the callus mass for each breeding line.

Improvement of the efficiencies of somatic embryo formation

Callus pieces taken from each embryogenic callus in some breeding lines with a relatively low frequency (<20% PSE) of somatic embryo formation were cultured on the somatic embryo-induction medium and on altered somatic embryo-induction media in which BAP was replaced by 1.0 mg l^{-1} of thidiazuron (TDZ), zeatin, or forchlorfenuron (N-(2-chloro-4-pyridyl)-N′-phenylurea, CPPU) and grown under the same conditions. After about 1 month, we examined whether somatic

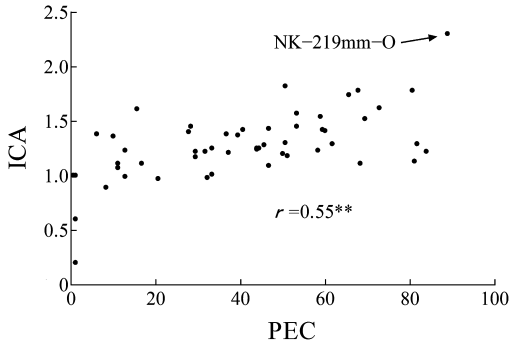

Figure 1. Relationship between the percentage of embryogenic callus formation (PEC) and the index of the callus amount (ICA) for cultured leaf and petiole explants in sugar beet breeding lines with PEC>0%. ** Indicate significance at $p<0.01$. ICA values: 0.2, for a diameter of the callus mass <1 mm; 1, 1 to <5 mm; 2, 5 to <10 mm; 3, 10 to <15 mm; 4, ≥15 mm.

embryos had formed from each callus, and also counted the number of somatic embryos per breeding line.

Data analysis

We calculated the correlations between pairs of variables (PEC and ICA, PSE and NSE, PEC and PSE) using Spearman's rank-correlation coefficient (r). Differences between treatments were tested for significance using Steel's multiple-comparison test.

Results

Embryogenic callus formation from leaf and petiole explants

In our preliminary experiments, the embryogenic calli induced from sugar beet leaf or petiole explants were white (or slightly brown) and friable, and were composed of round or somewhat packed cells. Using the 61 breeding lines, we examined whether such an embryogenic callus formed on each piece of leaf or petiole after culturing the explants for about 2 months on the callus-induction medium. The amount of callus that formed on each explant was scored using ICA. In this analysis, we excluded calli composed of compact cells or a mass of separated elongated cells. Table 1 summarizes the mean PEC and ICA values for each breeding line. PEC varied widely among the lines, ranging from 0 to 88.9%, and ICA varied from 0.2 to 2.3. ICA increased with increasing PEC, and there was a significant correlation between them ($r=0.55$, $p<0.01$; Figure 1). The breeding line NK-219 mm-O had the highest PEC and ICA values.

Somatic embryo formation from embryogenic callus

We counted the number of somatic embryos per callus after about 1 month of culturing the embryogenic callus on the somatic embryo-induction medium. The shape of the somatic embryos could be described as globular, heart-shaped, or torpedo-shaped (Figure 2); extremely deformed embryos were excluded from this count. Table 2 summarizes the mean PSE and NSE values for each breeding line. Considerable differences in PSE existed among the breeding lines, with PSE ranging from 0 to 98.9%. As in the callus formation analysis, NSE tended to increase with increasing PSE, especially for PSE >60%, and there was a strong and significant correlation between PSE and NSE ($r=0.86$, $p<0.01$; Figure 3). It is noteworthy that some breeding lines had PSE and NSE comparable to those of NK-219 mm-O.

Figure 2. Somatic embryos formed after about 1 month of culturing the embryogenic callus on the somatic embryo-induction medium.

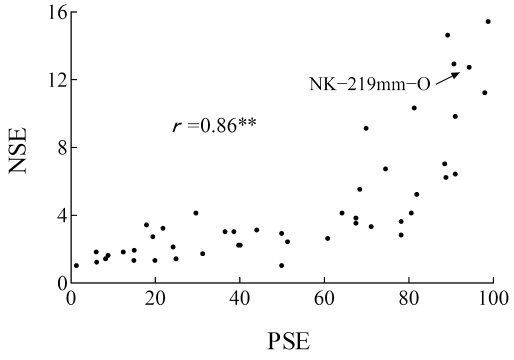

Figure 3. Relationship between the percentage of somatic embryo formation (PSE) and the number of somatic embryos per callus (NSE) for cultured embryogenic calli of the sugar beet breeding lines. ** Indicate significance at $p<0.01$.

An average of 30 to 40% of these somatic embryos continued to grow into plantlets when they were subcultured on the basal medium supplemented with 0.25 mg l^{-1} BAP (data not shown).

Improvement in PEC and ICA

When cotyledon explants were cultured on the callus-induction medium, they showed a higher PEC than the leaf and petiole explants in most breeding lines (Table 3). ICA also generally increased for the cotyledon explants. However, in breeding lines with a PEC of 0% for the leaf and petiole explants, only cotyledon explants from NK-300 mm-O produced the calli. On the other hand, when hypocotyl explants were cultured instead of leaf

Table 2. The percentage of somatic embryo formation (PSE) and the number of somatic embryos per callus (NSE) for embryogenic calli of the sugar beet breeding lines.

Breeding line	PSE (%)	NSE[a]	Breeding line	PSE (%)	NSE[a]
NK-204mm-O	0±0	—	NK-307mm-O	44.1±4.6	3.1±0.4
NK-214mm-O	0±0	—	NK-290BRmm-O	50.0±35.4	1.0±0
NK-221mm-O	0±0	—	NK-313mm-O	50.0±6.6	2.9±0.4
NK-229BRmm-O	0±0	—	NK-230BRmm-O	51.4±4.7	2.4±0.3
NK-233mm-O	0±0	—	NK-311mm-O	60.9±10.2	2.6±0.6
NK-245BRmm-O	0±0	—	NK-244BRmm-O	64.3±9.1	4.1±0.9
NK-329mm-O	0±0	—	NK-256BRmm-O	67.6±4.5	3.5±0.4
NK-294mm-O	1.4±1.4	1.0±0	NK-295BRmm-O	67.6±4.6	3.8±0.3
NK-205mm-O	6.1±2.0	1.8±0.5	NK-183BRmm-O	68.5±6.3	5.5±0.7
NK-220BRmm-O	6.2±2.0	1.2±0.1	NK-323mm-O	70.0±8.4	9.1±1.4
NK-234BRmm-O	8.3±3.6	1.4±0.4	NK-255BRmm-O	71.2±5.6	3.3±0.4
NK-242mm-O	8.9±3.2	1.6±0.4	NK-182mm-O	74.6±5.3	6.7±0.8
NK-235BRmm-O	12.5±3.7	1.8±0.3	NK-254BRmm-O	78.3±8.6	2.8±0.5
NK-324mm-O	15.0±8.0	1.3±0.3	NK-306mm-O	78.3±5.3	3.6±0.4
NK-196mm-O	15.1±4.9	1.9±0.6	NK-207mm-O	80.7±3.3	4.1±0.3
NK-227BRmm-O	18.0±5.4	3.4±1.0	NK-328mm-O	81.4±4.6	10.3±0.9
NK-215mm-O	19.5±3.6	2.7±0.7	NK-236BRmm-O	82.0±3.1	5.2±0.4
NK-206mm-O	20.0±10.3	1.3±0.3	NK-291BRmm-O	88.6±7.4	7.0±0.6
NK-213BRmm-O	21.9±4.2	3.2±0.9	NK-195BRmm-O	88.9±3.4	6.2±1.6
NK-193mm-O	24.3±4.1	2.1±0.4	NK-180BRmm-O	89.3±2.9	14.6±0.7
NK-301mm-O	25.0±9.7	1.4±0.4	NK-327mm-O	90.8±2.5	12.9±0.6
NK-310mm-O	29.7±4.8	4.1±0.7	NK-190mm-O	91.1±3.0	9.8±0.6
NK-226BRmm-O	31.3±4.7	1.7±0.2	NK-326mm-O	91.1±3.2	6.4±0.6
NK-184mm-O	36.6±5.3	3.0±0.5	NK-219mm-O	94.4±1.8	12.7±0.5
NK-299mm-O	38.7±4.7	3.0±0.4	NK-191mm-O	98.1±1.9	11.2±1.0
NK-197mm-O	39.8±5.4	2.2±0.3	NK-181BRmm-O	98.9±1.1	15.4±0.8
NK-203mm-O	40.2±5.1	2.2±0.3			

[a] Mean number of somatic embryos that formed on each callus. Values are the mean±SE.

Table 3. The percentage of embryogenic callus formation (PEC) and the index of the callus amount (ICA) for the different explant types from the sugar beet breeding lines[a].

Breeding line	PEC (%)			ICA[b]		
	Leaf and petiole explants	Cotyledon explants	Hypocotyl explants	Leaf and petiole explants	Cotyledon explants	Hypocotyl explants
NK-208BRmm-O	0±0	0±0	0±0	—	—	—
NK-239BRmm-O	0±0	0±0	0±0	—	—	—
NK-246BRmm-O	0±0	0±0	0±0	—	—	—
NK-248BRmm-O	0±0	0±0	0±0	—	—	—
NK-300mm-O	0±0	8.3±8.0	8.3±8.0	—	1.3±0.1	1.0±0.1
NK-245BRmm-O	0.6±0.5	8.3±6.1	0±0	1.0±0	1.8±0.2	—
NK-229BRmm-O	1.1±0.8	6.3±3.1	0±0	0.2±0	1.0±0.1	—
NK-329mm-O	1.1±0.8	0±0	0±0	0.6±0	—	—
NK-206mm-O	8.3±3.0	31.3±10.3	12.5±6.3	0.9±0.1	0.9±0.1	1.0±0.1
NK-195BRmm-O	10.0±3.7	29.2±11.0	4.2±4.0	1.4±0.1	1.4±0.2	2.0±0.2
NK-301mm-O	11.1±3.0	35.4±11.2	0±0*	1.1±0.1	1.0±0.1	—
NK-324mm-O	11.1±2.6	16.7±9.0	0±0*	1.1±0.1	2.3±0.3**	—
NK-254BRmm-O	12.8±3.2	47.9±14.0	12.5±6.3	1.2±0.1	1.9±0.3**	1.3±0.2
NK-311mm-O	12.8±4.8	33.3±13.6	12.5±6.3	1.0±0.1	1.9±0.3**	0.7±0.1
NK-244BRmm-O	15.6±4.7	43.8±13.9	16.7±6.8	1.6±0.1	2.3±0.3**	1.3±0.2
NK-323mm-O	16.7±2.8	77.1±9.1**	16.7±9.0	1.1±0.1	1.5±0.2	1.3±0.1
NK-233mm-O	20.6±2.9	91.7±4.5**	0±0**	1.0±0.1	2.5±0.1**	—
NK-227BRmm-O	27.8±6.2	66.7±13.6	33.3±13.6	1.4±0.1	2.8±0.4**	1.6±0.2
NK-221mm-O	28.3±6.8	50.0±14.4	50.0±14.4	1.5±0.1	2.6±0.4**	2.4±0.4**
NK-191mm-O	29.4±4.6	93.8±3.1**	54.2±12.4	1.2±0.1	1.8±0.1**	1.7±0.3*
NK-196mm-O	29.4±4.5	100±0**	54.2±11.0	1.2±0.1	2.0±0.1**	1.8±0.2**
Average	11.3	35.2	13.3	0.8	1.4	0.8

[a] * and ** indicate a significant difference compared with the values for the leaf and petiole explants at $p<0.05$ and $p<0.01$, respectively. [b] Values of ICA are defined based on the diameter of the callus mass: 0.2, <1 mm; 1, 1 to <5 mm; 2, 5 to <10 mm; 3, 10 to <15 mm; 4, ≥15 mm. Values are the mean±SE.

Table 4. Effects of different cytokinins or plant growth regulators with cytokinin activity on somatic embryo formation in sugar beet breeding lines that showed relatively low frequencies (<20% PSE) of somatic embryo formation.

Breeding line	Number of cultured embryogenic calli	Number of calli that formed somatic embryos (total number of somatic embryos in brackets)			
		BAP	TDZ	Zeatin	CPPU
NK-196mm-O	1	1 (3)	1 (3)	1 (1)	0
NK-204mm-O	12	0	2 (5)	0	1 (2)
NK-205mm-O	2	0	0	1 (2)	0
NK-215mm-O	7	1 (6)	2 (13)	0	1 (7)
NK-220BRmm-O	6	0	1 (1)	0	0
NK-221mm-O	7	0	3 (3)	0	0
NK-294mm-O	6	0	0	0	0
Total	41	2 (9)	9 (25)	2 (3)	2 (9)

and petiole explants, definite (but not always statistically significant) increases in PEC and ICA were observed in only four breeding lines (NK-300 mm-O, NK-221 mm-O, NK-191 mm-O, and NK-196 mm-O). No callus formation from hypocotyl explants was observed in three breeding lines whose leaf and petiole explants generated calli with PEC >10% (NK-301 mm-O, NK-324 mm-O, and NK-233 mm-O). As a result, the average PEC and ICA for hypocotyl explants were similar to those for leaf and petiole explants.

Improvement in PSE and NSE

PSE was higher on the altered somatic embryo-induction medium supplemented with TDZ than on the standard medium with BAP in the breeding lines that showed relatively low PSE values, but the difference was not statistically significant, and the altered media containing zeatin or CPPU did not increase embryo formation (Table 4). It was noteworthy that the altered medium containing TDZ caused the formation of a few somatic embryos in some breeding lines that did not form somatic embryos on the standard medium containing BAP.

Discussion

We examined the regeneration efficiencies for somatic embryogenesis in 61 sugar beet breeding lines using a previously reported procedure. These breeding lines are inbred lines that have considerable genetic diversity derived from some excellent ancestor varieties that are suitable for the cultural conditions in Japan, and they can be used to produce F_1 hybrids (Taguchi et al. 2006b). Taguchi et al. (2007) also reported that some of these lines showed moderate or better resistance to three major diseases that are problems for sugar beet in Japan: Cercospora leaf spot, Rhizoctonia root rot, and Aphanomyces root rot. In this study, we used leaf and petiole explants from seedlings, even though Tamagake (1999) reported that hypocotyl explants were more effective for callus induction. We found that PEC varied widely, ranging from 0 to 88.9%, with relatively

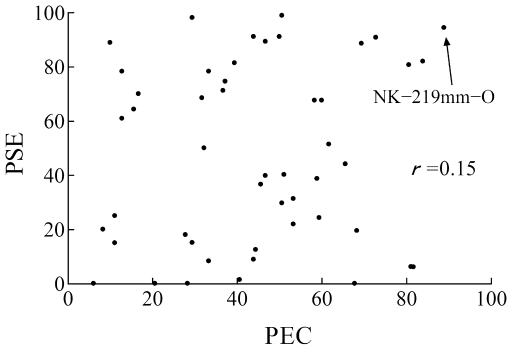

Figure 4. Relationship between the percentage of embryogenic callus formation (PEC) and the percentage of somatic embryo formation (PSE) for sugar beet breeding lines with PEC >1.1%. The breeding lines with PEC ≤1.1% generally formed a callus mass smaller than 3 mm, so their PSE may not reflect the correct values.

continuous variation (Table 1). It was previously recognized that the frequencies of embryogenic callus formation in sugar beet cultivars and breeding lines depended on the kinds of explants that were used (Roussy et al. 1996; Zhang et al. 2004) or their genotypes (Abe et al. 1991; Doley and Saunders 1989; Saunders and Shin 1986; Yu 1989; Zhang et al. 2004, 2008). These results may reflect differences in the levels of endogenous plant hormones in the explants or in various genetic factors that control callus formation in the breeding lines.

Similarly, PSE varied widely, ranging from 0 to 98.9% in the 53 breeding lines that formed embryogenic calli, and these results also showed relatively continuous variation (Table 2). The frequencies of somatic embryo formation were previously shown to vary among genotypes in sugar beet cultivars and breeding lines (Abe et al. 1991; Doley and Saunders 1989; Saunders and Shin 1986; Zhang et al. 2008), so these results may reflect genetic differences, as we hypothesized for the callus formation. There was no significant correlation between PEC and PSE (r=0.15; Figure 4). Dovzhenko and Koop (2003) also reported that there was no correlation between the efficiency of callus formation and the regeneration capability of friable calli. These results suggest that different aspects of an accession's genetic background are responsible for callus formation and

Table 5. The index and evaluation of the capability for somatic embryogenesis for leaf and petiole explants of 61 sugar beet breeding lines[a].

Breeding line	PEC (%)	ICA	PSE (%)	NSE	Index of somatic embryogenesis capability[b]	Evaluation of somatic embryogenesis capability
NK-219mm-O	88.9	2.3	94.4	12.7	**24.43**	excellent
NK-327mm-O	72.8	1.6	90.8	12.9	**13.75**	
NK-181BRmm-O	50.6	1.3	98.9	15.4	**10.01**	
NK-180BRmm-O	46.7	1.4	89.3	14.6	**8.70**	good
NK-291BRmm-O	69.4	1.5	88.6	7.0	**6.58**	
NK-190mm-O	50.0	1.2	91.1	9.8	**5.34**	
NK-207mm-O	80.6	1.8	80.7	4.1	**4.71**	
NK-328mm-O	39.4	1.4	81.4	10.3	**4.55**	
NK-236BRmm-O	83.9	1.2	82.0	5.2	**4.34**	
NK-191mm-O	29.4	1.2	98.1	11.2	**3.78**	
NK-326mm-O	43.9	1.3	91.1	6.4	**3.20**	
NK-182mm-O	37.2	1.2	74.6	6.7	**2.25**	
NK-256BRmm-O	60.0	1.4	67.6	3.5	**2.01**	
NK-295BRmm-O	58.3	1.2	67.6	3.8	**1.82**	
NK-307mm-O	65.6	1.7	44.1	3.1	**1.55**	
NK-183BRmm-O	31.7	1.2	68.5	5.5	**1.46**	
NK-323mm-O	16.7	1.1	70.0	9.1	**1.18**	
NK-255BRmm-O	36.7	1.4	71.2	3.3	**1.18**	
NK-310mm-O	50.6	1.8	29.7	4.1	**1.11**	
NK-299mm-O	58.9	1.5	38.7	3.0	**1.04**	
NK-230BRmm-O	61.7	1.3	51.4	2.4	**1.00**	
NK-306mm-O	33.3	1.0	78.3	3.6	**0.95**	fair
NK-195BRmm-O	10.0	1.4	88.9	6.2	**0.75**	
NK-244BRmm-O	15.6	1.6	64.3	4.1	**0.65**	
NK-184mm-O	45.6	1.3	36.6	3.0	**0.63**	
NK-213BRmm-O	53.3	1.6	21.9	3.2	**0.59**	
NK-203mm-O	51.1	1.2	40.2	2.2	**0.55**	
NK-313mm-O	32.2	1.0	50.0	2.9	**0.46**	
NK-197mm-O	46.7	1.1	39.8	2.2	**0.45**	
NK-193mm-O	59.4	1.4	24.3	2.1	**0.43**	
NK-226BRmm-O	53.3	1.5	31.3	1.7	**0.41**	
NK-215mm-O	68.3	1.1	19.5	2.7	**0.39**	
NK-254BRmm-O	12.8	1.2	78.3	2.8	**0.35**	
NK-227BRmm-O	27.8	1.4	18.0	3.4	**0.24**	
NK-311mm-O	12.8	1.0	60.9	2.6	**0.20**	
NK-235BRmm-O	44.4	1.3	12.5	1.8	**0.12**	
NK-205mm-O	81.7	1.3	6.1	1.8	**0.11**	
NK-196mm-O	29.4	1.2	15.1	1.9	**0.10**	
NK-242mm-O	43.9	1.2	8.9	1.6	**0.08**	poor
NK-220BRmm-O	81.1	1.1	6.2	1.2	**0.07**	
NK-234BRmm-O	33.3	1.3	8.3	1.4	**0.05**	
NK-301mm-O	11.1	1.1	25.0	1.4	**0.04**	
NK-324mm-O	11.1	1.1	15.0	1.3	**0.02**	
NK-206mm-O	8.3	0.9	20.0	1.3	**0.02**	
NK-294mm-O	40.6	1.4	1.4	1.0	**0.01**	
NK-290BRmm-O	1.1	1.0	50.0	1.0	**0.01**	
NK-204mm-O	67.8	1.8	0	—	**0**	
NK-221mm-O	28.3	1.5	0	—	**0**	
NK-233mm-O	20.6	1.0	0	—	**0**	
NK-214mm-O	6.1	1.4	0	—	**0**	
NK-229BRmm-O	1.1	0.2	0	—	**0**	
NK-329mm-O	1.1	0.6	0	—	**0**	
NK-245BRmm-O	0.6	1.0	0	—	**0**	
NK-185BRmm-O	0	—	—	—	**0**	
NK-194mm-O	0	—	—	—	**0**	
NK-208BRmm-O	0	—	—	—	**0**	
NK-237BRmm-O	0	—	—	—	**0**	
NK-239BRmm-O	0	—	—	—	**0**	
NK-246BRmm-O	0	—	—	—	**0**	
NK-248BRmm-O	0	—	—	—	**0**	
NK-300mm-O	0	—	—	—	**0**	

[a] PEC, percentage of embryogenic callus formation; ICA, index of the callus amount; PSE, percentage of somatic embryo formation; NSE, number of somatic embryos per callus. Values of ICA are defined based on the diameter of the callus mass: 0.2, <1 mm; 1, 1 to <5 mm; 2, 5 to <10 mm; 3, 10 to <15 mm; 4, ≥15 mm. [b] This index was calculated as (PEC/100)×ICA×(PSE/100)×NSE. Values are means.

somatic embryo formation. However, the breeding line NK-219 mm-O showed both high PEC and high PSE (Figure 4), suggesting that this breeding line may have a useful genetic background for both callus formation and somatic embryo formation. Values of the genetic contribution from seven major ancestral populations to the breeding lines examined in this study were reported by Taguchi et al. (2006a), who showed that NK-219 mm-O contained the same genetic contribution from two ancestral populations as NK-220 mm-O and NK-221 mm-O, both of which had low PSE (<10%). Therefore, it is difficult to clarify the genetic origin of high PEC and high PSE in NK-219 mm-O based solely on the line's pedigree.

PEC and PSE alone cannot adequately describe the capability for somatic embryogenesis in each breeding line because they do not include a measure of the callus amount or the number of somatic embryos per callus. To solve this problem, we developed an index of the capability for somatic embryogenesis:

$$\text{Index of somatic embryogenesis capability} = (\text{PEC}/100) \times \text{ICA} \times (\text{PSE}/100) \times \text{NSE}$$

The index values ranged from 0 to 24.4 among the breeding lines based on the parameter values for leaf and petiole explants (Table 5). These values represent the expected number of somatic embryos per explant. Though these values cannot precisely indicate each breeding line's capability for somatic embryogenesis (because each of the four parameters used to calculate the index contains its own error term), the index can nonetheless be used to roughly classify the 61 breeding lines. Using the index values, we classified the breeding lines into four groups: those with an index ≥10 were rated as excellent, those with 1≤ index <10 were rated as good, those with 0.1≤ index <1 were rated as fair, and those with an index <0.1 were rated as poor. This classification showed that 3 breeding lines were excellent, 18 breeding lines were good, 17 breeding lines were fair, and 23 breeding lines were poor (Table 5). None of the breeding lines had a capability for somatic embryogenesis as high as that of breeding line NK-219 mm-O, but NK-327 mm-O and NK-181BRmm-O had relatively high capabilities. The 21 breeding lines that were rated as excellent or good are expected to produce one or more somatic embryos per explant, and are thus suitable for use in *Agrobacterium*-mediated transformation via somatic embryogenesis. This group included four breeding lines (NK-255BRmm-O, NK-256BRmm-O, NK-291BRmm-O, and NK-295BRmm-O) that showed moderate or better resistance to the three major diseases described above, and one breeding line (NK-310 mm-O) that showed strong resistance to these diseases (Taguchi et al. 2007). Thus, they are useful materials for developing transgenic breeding lines because of their superior characteristics. On the other hand, the 40 breeding lines with an index value <1.0 will be difficult to use for somatic embryogenesis using currently available techniques.

Because it is desirable to use breeding lines with diverse genetic backgrounds for *Agrobacterium*-mediated transformation via somatic embryogenesis, it is necessary to increase the efficiency of embryogenic callus and somatic embryo formation in these 40 lines. We observed a weak but significant positive correlation between PEC and ICA (Figure 1) and a stronger significant correlation between PSE and NSE (Figure 3); this suggests that the efficiency of somatic embryogenesis could be increased by increasing the values of any of these four parameters. We therefore examined the effects of the explant type and of the plant growth regulator used in the somatic embryo-induction medium.

Gürel et al. (2001) reported that cotyledon and hypocotyl explants produced significantly more calli than petiole and leaf explants, so we compared the PEC of cotyledon and hypocotyl explants with that of leaf and petiole explants. We found that cotyledon explants produced a higher PEC in many of the breeding lines (Table 3). However, Krens and Jamar (1989) found that the frequencies of callus formation from seedling explants were affected by the BAP concentrations in the culture media, so the improvements in PEC obtained by using cotyledon explants may be limited to the culture conditions used in the present experiment. Furthermore, ICA increased in many of the breeding lines when cotyledon explants were used (Table 3), so using cotyledon explants appears to be an effective means for improving the callus formation efficiency in the sugar beet breeding lines used in this experiment. However, for most of the breeding lines with 0% PEC, using cotyledon explants did not improve the callus formation efficiency, indicating that further research will be needed to efficiently induce the calli in these breeding lines.

In breeding lines with relatively low PSE, adding TDZ instead of BAP to the somatic embryo-induction medium generally increased PSE and NSE (Table 4). Zhang et al. (2001) showed that TDZ was more effective than BAP for the promotion of adventitious shoot formation in sugar beet, but the TDZ treatment was not always superior to the BAP treatment and the improvement provided by TDZ depended upon the genotype. Dovzhenko and Koop (2003) showed that no regeneration occurred in sugar beet cultured on some media supplemented with TDZ, and suggested that TDZ was not useful for sugar beet regeneration in all tissues because the response to TDZ differed among the type of explants and among the calli. The breeding lines examined in the present experiment were generally responsive to TDZ. Some breeding lines that showed 0% PSE on the medium with BAP produced somatic

embryos on the medium containing TDZ. We therefore conclude that it is worthwhile testing TDZ as a possible means of improving the efficiency of somatic embryo formation in sugar beet breeding lines.

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