First Report of Adventitious Shoots from Intact Sugarbeet Plants and Its Implications

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Abstract

Simple and efficient genetic transformation in sugarbeet has long been unavailable because of the absence of a satisfactory technology for the direct (i.e., not involving callus) *de novo* formation of shoots from leaves (or parts thereof). However, of course, such a system has long been available for use with *Rhizobium* (formerly *Agrobacterium*) transformation of tobacco, for example. Labs have reported the formation of adventitious shoots from *in vitro* grown sugarbeet shoots and seedlings, or from leaf pieces and thin cell layers from these. Since these adventitious shoots presumably originated from pre-formed meristematic 'initials' induced during the prior *in vitro* culture of the donor shoots and seedlings, they have not been considered amenable for either direct selection or genetic transformation. Currently, we have obtained direct adventitious shoots in a one step procedure using leaf pieces of greenhouse-grown plants sugarbeet clone REL-1. Up to 83% of leaf pieces regenerated one or more shoots with single midvein pieces one-to-two cm long initially placed on semi-solid Murashige-Skoog media with 1 mg/L N^6-benzyladenine and then maintained at 23-24^0 C for seven-to-twelve weeks in low light intensity light from overhead fluorescent lamps. This new discovery will likely provide for the simple and efficient regeneration that has long been needed for genetic transformation of sugarbeet.

Introduction

Intensive efforts have been made to develop tissue culture systems useful for genetic improvement of the sugarbeet and, albeit with the development of some applications such as micropropagation (Saunders, 1982; Zhong et al, 1993), somatic cell selection (Saunders et al., 1992) and a relatively inefficient genetic transformation system (Snyder et al, 1999). Progress in developing a simple, direct and efficient regeneration protocol suitable for genetic transformation of sugarbeet has nevertheless been limited. Adventitious shoot formation without intermediate callus in sugarbeet has been described, for example adventitious shoots appeared after a time on some leaves that subsequently developed on intact potted plants where the seedling apex had earlier been treated with the cytokinin N^6-benzyladenine (Saunders and Mahoney, 1982). *In vitro* adventitious shoots have been reported on leaves of axenic shoot cultures (Saunders, 1982; Harms et al, 1983; Hussey and Hepher, 1978), and on explants of leaves or petioles taken from *in* vitro-cultivated seedlings or shoots (Rogozinska and Goska, 1978, Detrez et al, 1988, 1989; Freytag et al, 1988; Hussey and Hepher, 1978; Ritchie et al, 1989; Saunders and Shin, 1986; Sabir and Ford-Lloyd, 1991; Grieve et al, 1997). In most cases, shoots arose on the adaxial surface of the petiole or the blade-petiole transition zone. In cultures of explanted petioles, shoots appeared as early as six days after initiation of the culture (Freytag et al, 1988). Adventitious shoots were also obtained from thin layer explants from seedlings grown *in vitro* with N^6-benzyladenine (Detrez et al, 1988; Toldi et al, 1996). However, none of these observations of adventitious shoots on leaf pieces from *in vitro* grown shoots or seedlings has been adapted for genetic

transformation; either because the shoots were believed to have arisen from internally preformed initials, or because of problems with either repeatibility or reproduciblity.

However, despite much effort over more than twenty years, direct adventitious shoots induced on leaf pieces taken from either field-grown or greenhouse-grown sugarbeet plants has not been reported. We now report a direct and simple means to obtain efficient regeneration of REL-1 sugarbeets without callous formation being involved.

Materials and Methods

Leaves were first surface-sterilized using two washes for 15min. of 15% Chlorox and 0.01% SDS after which tissue is rinsed five times with sterile water. Single 1.5 cm pieces of either petiole or midvein (from the lower half of the leaf blade) were then placed on the surface of either of 10 different experimental media composed as listed in the tables. Media contained N^6-benzyladenine (BA) and K-naphthaleneacetic acid (NAA), either singularly or in combination, in a base medium of modified Murashige-Skoog (1962) inorganic salts medium with 30 g/L sucrose, 100 mg/L *myo*-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine-HCl, 0.1 mg/L thiamine-HCl, 0.5 g/L 2-(N-morpholino)ethanesulfonic acid and 5 g/L Agargel (Sigma Chemical Co, St. Louis Missouri USA), where the pH had been adjusted to about 5.8 with KOH prior to autoclaving. Media were dispensed after autoclaving into aliqouts of about 35 ml per 100 x 20 mm disposable plastic Petri dish. After the transfer of surface-disinfected petiole or midvein leaf pieces, plates were then double-sealed with strips of Parafilm to reduce dessication (mention of a tradename does not imply endorsement). Plates were kept at the ambient laboratory temperature (23.5^0 C) in stacks in dim light about a meter from Cool White fluorescent bulbs.

Results and Discussion

Evaluation of explant response after six weeks revealed neither callus nor adventitious shoots of any kind. Evaluation after 15 weeks revealed seven explants each with single adventitious shoots growing directly from one end (Table 2), in most cases clearly from the basipetal ends of the explants. One midvein explant on MS plus 1.0 mg/L BA had three adventitious shoots growing from one end. Five of the eight explants with adventitious shoots were on medium with 1.0 mg/L BA as the sole growth regulator, the remaining three on media with 1.0 mg/L BA plus NAA (Table 1). Five of the six explants on B1 medium gave rise to attached shoots, a 83% success rate!

Thirteen of the explants had given rise to soft whitish, often detached, callus with buds or shoots on it. None of these explants, however, had shoots directly derived from the explant (Table 1). Other explant responses present were 'loose callus only' (i.e., no buds or shoots), 'hard green attached callus only', 'rootlets only' (up to several cm long), and 'no response'.

Despite the relatively low number of explants put on each experimental medium, it was clear that attached adventitious shoot formation occurred in most cases in the absence of exogenous auxin (i.e., on media without NAA), and exclusively on media initially containing 1.0 mg/L BA, and predominantly on media including 1.0 mg/L BA in the absence of NAA (Table 1). There was also clearly an absence of bud and shoot formation on any loose (i.e., whitish, hormone autonomous) callus present (Table 1).

The adventitious shoots on these explants from petioles and midveins arose from the ends of the explants, quite unlike the adventitious shoots that have been reported arising in lengthwise manner from the interior of petioles and petiole-blade transition zones of leaves and petiole

explants from in vitro cultured shoots and seedlings. Another difference between the adventitious shoots reported here and those that have developed from leaves and leaf pieces from *in vitro* cultured shoots and seedlings is the slowness to appear of the shoots reported here: seven to twelve weeks compared with the several weeks generally noted from such *in vitro* explants.

Table 1.

MIDVEIN EXPLANTS Medium	Attached shoot only	Attached shoot with callus only	Only loose callus with shoots or buds	Hard green callus only	No Response
M0					2 of 2
N.1					2 of 2
N.3					2 of 2
B.3			1 of 2	1 of 2	
B1	3 of 4	1 of 4			
B1N.03			2 of 2		
B1N.1			1 of 1		
B1N.3	1 of 2		1 of 2		
B1N3	1 of 3		1 of 3	1 of-3	
B2			2 of 2		

This slowness to develop may account for the absence of other reports of adventitious shoots on explants from intact plant leaves, as in most cases investigators would have discarded such leaf explant cultures after five or six weeks, before visible shoots developed.

The single genotype used in this research, REL-1, is a germplasm developed and subsequently used in applications of an unconventional callus induction, shoot regeneration, and somatic embryo system (Saunders 1982, Saunders and Doley 1986, Doley and Saunders 1989, Saunders, 1998) based on temperature-dependent delayed appearance of high-frequency hormone-autonomous callus from leaf piece explants. Interestingly, the 1.0 mg/L BA in MS medium that was most effective in inducing direct adventitious shoots in this report is also the BA concentration previously considered optimal for use in eliciting hormone-autonomous callus and subsequent shoot regeneration. At the six week post-inoculation time reported here at 23-24^0 C, there was no callus present, but loose callus was reported from explants on all BA-containing media at 15 weeks.

This study describing experiments achieving direct adventitious shoots from leaf explants

from intact sugarbeet plants is the first report of such adventitious shoots, and this new discovery is expected to be of great usefulness as it will probably lead to development of a leaf disc procedure for simple and efficient genetic transformation of sugarbeet. Adventitious shoots on sugarbeet discs of leaf laminar tissue have also been obtained using MS plus 1.0 mg/L BA in a similar one-step procedure.

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References cited

Detrez C, Sangwan RS, Sangwan-Norreel BS. 1989. Phenotypic and karyotypic status of *Beta vulgaris* plants regenerated from direct organogenesis in petiole culture. Theor. Appl. Genet. 77: 462-468.

Detrez C, Tetu T, Sangwan RS, Sangwan-Norreel BS. 1988. Direct organogenesis from petiole and thin cell layer explants in sugar beet cultured in vitro. J. Expl. Bot. 39: 917-926.

Doley WP, Saunders JW. 1989. Hormone-free medium will support callus production and subsequent shoot regeneration from whole plant leaf explants in some sugarbeet (*Beta vulgaris* L.) populations. Plant Cell Rep. 8: 222-225.

Freytag AH, Anand SC, Rao-Arelli AP, Owens LD. 1988. An improved medium for adventitious shoot formation and callus induction in *Beta vulgaris* L. *in vitro*. Plant Cell Rep. 7: 30-34.

Grieve TM, Gartland KMA, Elliott MC. 1997. Micropropagation of commercially important sugar beet cultivars. Plant Growth Regul. 21.

Harms CT, Baktir I, Oertli JJ. 1983. Clonal propagation in vitro of red beet (*Beta vulgaris* ssp) by multiple adventitious shoot formation. Plant Cell Tis. Org. Cult. 2: 93-102.

Hussey G, Hepher A. 1978. Clonal propagation of sugar beet plants and the formation of polyploids by tissue culture. Ann. Bot. 42: 477-479.

Ritchie GA, Short KC, Davey MR. 1989. In vitro shoot regeneration from callus, leaf axils and petioles of sugar beet (*Beta vulgaris* L.). J. Exp. Bot. 40: 277-283.

Rogozinska J, Goska M. 1978. Induction of differentiation and plant formation in isolated sugar beet leaves. Bull. Acad. Pol. Sci. (Biol.) 26: 343-345.

Sabir, AA, Ford-Lloyd BV. 1991. Processing crop germplasm in vitro for mass production of regenerants: a case study with beet. J. Biotechnol 17: 257-268.

Saunders JW. 1982. A flexible *in vitro* shoot culture propagation system for sugarbeet that includes rapid floral induction of ramets. Crop Sci. 22: 1102-1105.

Saunders JW, Mahoney MD. 1982. Benzyladenine induces foliar adventitious shoot formation on young plants of two sugarbeet (*Beta vulgaris* L.) cultivars. Euphytica 31: 801-804.

Saunders JW, Doley WP. 1986. One-step shoot regeneration from callus of whole plant leaf explants of sugarbeet lines and a somaclonal variant for in vitro behaviour. J. Plant Physiol. 124:473-479.

Saunders JW, Shin K. 1986. Germplasm and physiologic effects on induction of high-frequency hormone autonomous callus and subsequent shoot regeneration in sugarbeet. Crop Sci. 26: 1240-1245.

Saunders JW., Acquaah, G., Renner, K.A, Doley, WP. 1992. Monogenic dominant sulfonylurea resistance sugarbeet (*Beta vulgaris*) from somatic cell selection. Crop Sci. 32:1357-1360.

Saunders JW. 1998. Registration of REL-1 and REL-2 sugarbeet germplasms for tissue culture genetic manipulations. Crop Sci. 38: 901-902.

Snyder GW, Ingersoll JC, Smigocki AC, Owens LD. 1999. Introduction of pathogen defense genes and a cytokinin biosynthesis gene into sugarbeet (*Beta vulgaris* L.) by *Agrobacterium* or particle bombardment. Plant Cell Rep. 18: 829-834.

Toldi O, Gyulai G, Kiss J, Tamas IA, Balazs E. 1996. Antiauxin enhanced microshoot initiation and plant regeneration from epicotyl-originated thin-layer explants of sugarbeet (*Beta vulgaris* L.). Plant Cell Rep. 15: 851-854.

Zhong ZX, Smith HG, Thomas TH. 1993. Micropropagation of wild beet (*Beta maritima*) from inflorescence pieces. Plant Growth Regul 12: 53-57.