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REVIEW

Stresses applied for the re-programming of plant microspores towards in vitro embryogenesis

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Microspore embryogenesis is the most commonly used method to produce doubled haploids. It is based on the ability of a single haploid cell, the microspore, to de-differentiate and regenerate into a whole plant after being exposed to stresses, such as low or high temperatures, carbon starvation and colchicine. Some stresses such as temperature treatments and carbon starvation have been used with success in many plant species, whereas others such as colchicine had limited application in a few species. Reports on the application of whole plant treatments with feminizing agents on inflorescences and buds are scarce. Furthermore, the technical means to apply some stresses such as γ-irradiation are not readily available. Recently, novel stresses such as pH, inducer chemicals, carrageenan oligosaccharides and heavy metals were reported to induce microspore embryogenesis. It remains to be seen, however, whether these stresses are effective in a wider range of species. Finally, pretreatment of cultured cells with high concentrations of 2,4-D efficiently induces somatic embryogenesis in several species (carrot, alfalfa). However, reports on the use of this particular chemical stress are not available in microspore embryogenesis. The paper presented here gives an overview of various stresses and mechanisms of action of these stresses in inducing microspore embryogenesis.

Stress as trigger to induce microspore embryogenesis

Among the different technologies to produce doubled haploids, microspore embryogenesis is by far the most common (Touraev et al. 2001). Other techniques are often restricted to a given species or genus. In cereals, the method of pollination with pollen from another cereal genus (*Hordeum bulbosum, Zea mays*) and embryo rescue is a potent rival of microspore-derived doubled haploids (Laurie and Bennett 1988, Inagaki 2003). In melons, pollination with irradiated pollen is commonly used (Sauton and Dumas-du-Vaulx 1987), whereas female gametes are the source of doubled haploids in sugar beet or onion (Hosemans and Bossoutrot 1983, Jakse and Bohanec 2003, Wremerth and Levall 2003).

Isolated and in vitro cultured plant cells under certain conditions regenerate into embryos and whole plants. This phenomenon is called 'totipotency'. Conversion of differentiated plant cells into totipotent cells depends on extraordinary conditions acting upon cells. A triggering factor in the form of stress is necessary to induce embryogenesis in cultured microspores (Nitsch and Norreel

Abbreviations – 2, 4-D, 2,4-dichlorophenoxy acetic acid; ABA, abscisic acid; HSP, heat-shock protein; IAA, indole-3-acetic acid; GA_3, gibberellic acid.

1973, Touraev et al. 1997). As part of remarkable survival strategy, in vitro cultured microspores, committed to develop into a pollen grain, leave the gametophytic pathway and resort to a sporophytic mode of development by producing haploid or doubled haploid embryos and plants (Fig. 1).

A number of early markers for the conversion of microspores towards embryogenesis have been described in the literature. Some of these markers, found in different species, i.e. *Nicotiana tabacum* (Dunwell and Sunderland 1974a, 1974b, Garrido et al. 1995, Touraev et al. 1996a), *Brassica napus* (Zaki and Dikinson 1990, Telmer et al. 1993), *Datura* (Sangwan and Camefort 1983, 1984), are (a) fragmentation of the vacuole by formation of cytoplasmic strands from the peri-nuclear to the subcortical cytoplasm; (b) movement of the nucleus to the centre of the microspore resulting in a central phragmosome; (c) increase in the size of the cell; (d) formation of a new cell wall below the exine; (e) size reduction of the nucleolus; (f) compaction of chromatin; (g) the appearance of a zone of multivesiculate bodies, resembling lysosomes and the degeneration of plastids; (h) tannin-coated tonoplasts; (i) size reduction of the starch grains; (k) no marked structural changes of mitochondria and (l) symmetric division with a planar wall instead of the regular asymmetric cell division in gametophytic microspores (for details see Touraev et al. 2001, Aionesei et al. 2005). But none of these markers were found to be universal in embryogenic microspores of all species. However, among them, the 'star-like' structure of a microspore after stress treatment, seen under the light microscope (Touraev et al. 1996a) and exhibiting a centralized nucleus surrounded by 'star-like' cytoplasmic strands was shown to be sign of embryogenic microspores in a number of different species such as wheat (Touraev et al. 1996b), tobacco (Touraev et al. 1996a), apple (Höfer et al. 1999) and rice (Raina and Irfan 1998). Furthermore, recent studies using tracking of the entire process of embryogenesis from single selected wheat microspores clarified that microspores with a star-like internal structure and a symmetrical cell division are essential for the initiation of the embryogenic development of isolated microspores (Indrianto et al. 2001). In other species, just the frequency of microspores with a symmetrical division is used as a marker (Telmer et al. 1993), although it has been shown that microspores with two cells of equal size can develop gametophytically (Touraev et al. 1995).

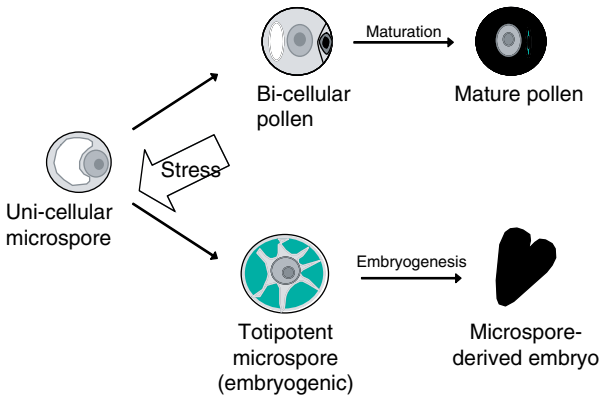

Fig. 1. Schematic presentation of the gametophytic and sporophytic pathways in microspores.

Microspores can be induced to become embryogenic either in planta, or in excised inflorescences or flower buds, or in in vitro cultured anthers or isolated microspores (Touraev et al. 1997). In all these cases, conversion to the sporophytic pathway can be induced by subjecting microspores to various stresses.

Another important factor for successful conversion is the stage at which inflorescences, flower buds, anthers or microspores are excised, put into in vitro culture or isolated from the anther, respectively. An important issue, however, is to find and choose the right conditions at a certain stage in a given species. For example, in tobacco, a heat treatment is only effective on microspores but not on bi-cellular pollen (Kyo and Harada 1986, Touraev et al. 1996a).

The available stresses can be categorized in different ways, e.g. according to the time of application (before or during the culture), type of stress (cold, heat, starvation), etc. In the present paper, we categorize stresses as widely used, neglected and novel (Fig. 2; Tables 1 and 2). A discussion along this line may help researchers to induce microspore embryogenesis in species hitherto proving recalcitrant.

Stresses widely used for the induction of microspore embryogenesis are cold, heat, carbon starvation and colchicine. The use of stress in the form of cold pretreatment was first reported by Nitsch and Norreel (1973) on *Datura* anthers or microspore cultures. Heat pretreatment is usually carried out at 33–37°C for a duration varying from several hours to several days, whereas cold treatment is carried out at 4–10°C from some days to several weeks. Incubation of microspores in media containing non-metabolizable carbon sources, i.e. in mannitol-containing media, is also used with success (Touraev et al. 2001). In addition, colchicine, a microtubule-depolymerizing agent, is being used as a stress pretreatment (Zaki and Dickinson 1991, Zhao et al. 1996).

The neglected stresses such as abscisic acid (ABA) (Imamura and Harada 1980a), feminizing agents (Heberle-Bors 1983), reduced atmospheric pressure

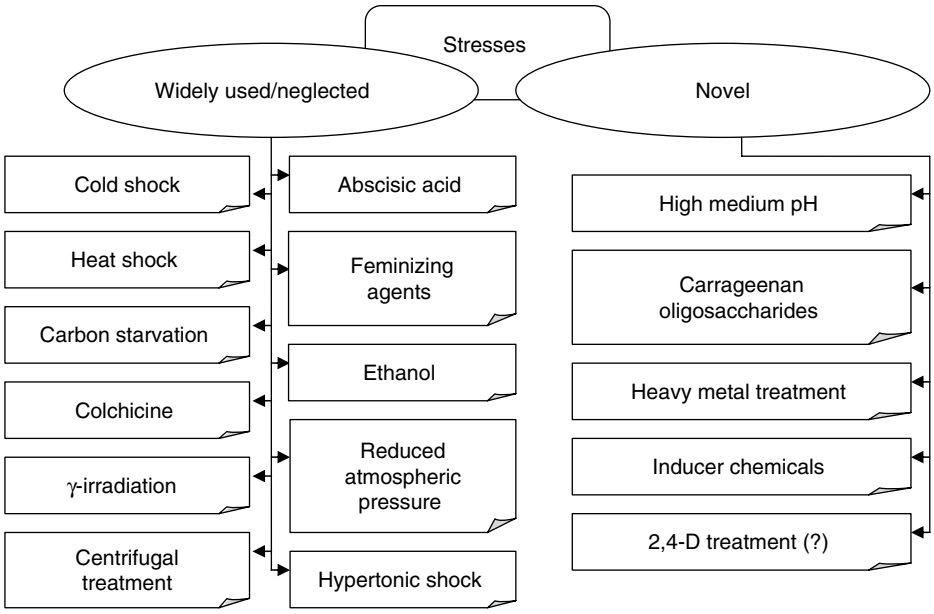

Fig. 2. Schematic presentation of widely used, neglected and novel stresses.

(Imamura and Harada 1980b), ethanol (Pechan and Keller 1989), hypertonic shock (Wang et al. 1981), centrifugal treatment (Tanaka 1973) and γ-irradiation (Sangwan and Sangwan 1986) have been tested only in few species. Hardly any recent reports widening the application of these stresses in other species are available. Scarcity of reports may be because, in general, widely used stresses, where applicable, are easier to handle and do not require extra labour. However, these stresses may prove effective to induce microspore embryogenesis in recalcitrant species in which the conventional stresses were not successful.

Recently, several novel stresses have been reported to induce microspore embryogenesis with success in some species. The incubation of microspores in a high medium pH (Barinova et al. 2004), or in the presence of inducer chemicals (Liu et al. 2001, Zheng et al. 2001), heavy metals (Zonia and Tupy 1995), carrageenan oligosaccharides (Lemonnier-Le Penhuizic et al. 2001) are all novel stresses to be tested in detail on other species.

Stresses widely used in microspore embryogenesis

Cold shock

Donor plants, excised spikes or flower buds and cultured anthers have been exposed to a low-temperature pretreatment to induce microspore embryogenesis (Touraev et al. 1997, 2001).

Tobacco plants grown at low temperature under the short-day condition contained anthers with a high number of sterile pollen, which showed the typical characteristics of P-grains (embryogenic pollen grains) (Heberle-Bors 1982a, 1982b, 1983). After separation from fertile pollen and in vitro culture, these P-grains developed into embryos (Heberle-Bors 1983).

Exposure of excised spikes and flower buds to low temperature-induced microspore embryogenesis in barley (Xu et al. 1981, Huang and Sunderland 1982), rice (Cho and Zapata 1988), bread wheat (Picard and De Buyser 1973, Gustafson et al. 1995), durum wheat (Labbani et al. 2005), oats (Kiviharju and Pehu 1998), triticale (Marciniak et al. 2003), *Citrus clementina* (Germana and Chiancone 2003) and flax (Obert et al. 2004). Cold shock applied to cultured anthers also induced microspore embryogenesis in species such as tobacco (Reinert et al. 1975, Duncan and Heberle 1976).

It was proposed that cold pretreatment slows down degradation processes in the anther tissues thus protecting microspores from toxic compounds released in the decaying anthers (Duncan and Heberle 1976). Cold assures survival of a greater proportion of the embryogenic pollen grains (Sunderland and Roberts 1979). Cold pretreatment also increases the frequency of endo-reduplication leading to an increase of spontaneously doubled haploid plants (Amssa et al. 1980). Microspores in cold-treated anthers disconnect from the tapetum resulting in starvation (Sunderland

Table 1. Protocols with special emphasis to widely used stress treatments on microspore embryogenesis in selected species. UC, uni-cellular; BC, bi-cellular; E, embryo; R, regenerant; C, callus; IMC, isolated microspore culture; AC, anther culture.

Plant	Stress	Protocol	Microspore stage	Result	System	Reference
Tobacco	Heat and starvation	33°C and B medium/6 days/ microspore suspension	Late UC	E&R	IMC	Touraev et al. (1996a)
Wheat	Heat and starvation	33°C and B medium/4 days/ anthers	Late UC-premitotic	E&R	IMC	Touraev et al. (1996b)
Brassica napus	Heat	32°C/72 h/microspore suspension	Late UC	E&R	IMC	Custers et al. (1994)
Broccoli	Heat	32.5°C/24 h/microspore suspension	Late UC-early BC	E&R	IMC	Dias (2003)
Barley	Starvation	Mannitol (0.7 *M*)/24°C/4 days/ anthers	Mid-late U	E&R	AC	Castillo et al. (2000)
Barley	Cold and/or starvation	+4°C and mannitol (0.3 *M*)/4 days/spikes +4°C/3–4 weeks/spikes mannitol (0.3 *M*)/25°C/4 days/ spikes	Mid-late UC	E&R	IMC	Kasha et al. (2001)
Barley	Cold	+4°C/2–4 weeks/spikes	Early-mid UC	E&R	IMC	Davies and Morton (1998)
Barley	Cold	+4°C/3–4 days/anthers	UC	E&R	AC	Jacquard et al. (2003)
Barley	Cold	4–5°C/21–28 days/spikes	Mid UC	C/E&R	AC	Szarejko (2003)
Wheat	Cold and/or starvation	+4°C and mannitol (0.4 *M*)/5–7 days/spikes +4°C/2–4 weeks/spikes mannitol (0.4 *M*)/25°C/5 days/ spikes	Mid-late UC	E&R	IMC	Kasha et al. (2003)
Wheat	Cold	+7°C/14 days/spikes	Early-mid UC	E&R	AC	Tuvesson et al. (2000)
Rice	Cold	8–10°C/8 days/panicles	Mid UC-early BC	C&R	AC	Zapata-Arias (2003)
Maize	Cold	+7°C/1 week/tassels	Mid UC	C/E&R	AC	Barnabas (2003b)
Triticale	Cold	+4°C/2 weeks/spikes	Mid-late UC	C/E&R	IMC	Pauk et al. (2000)
Triticale	Cold	+7°C/14 days/spikes	Early-mid UC	E&R	AC	Tuvesson et al. (2000)
Durum wheat	Cold	4°C/7 days/spikes	Mid UC	C/E&R	AC	Jauhar (2003)
Potato	Cold	4–6°C/72 h/buds	Late UC-early BC	C/E&R	AC	Aziz et al. (1999), Tai and Xiong (2003)
Apple	Cold and starvation	4°C/1–2 weeks/buds then 4°C and B med./2–3 days/microspore suspension	Late UC	E&R	IMC	Höfer et al. (1999)
Citrus	Cold	4°C/8–15 days/buds	Late UC	C&R	AC	Germana (2003)
B. napus	Colchicine	Colchicine (25 μ*M*)/42 h/25°C/ microspore suspension	Late UC	E&R	IMC	Zhao et al. (1996)
Wheat	Colchicine and mild heat	Colchicine (0.04%)/29°C/3 days/ anthers	Mid-late UC	C/E&R	AC	Barnabas (2003a)
Maize	Colchicine	Colchicine (0.02%)/3 days/ anthers	Mid UC	E&R	AC	Obert and Barnabas (2004)

and Xu 1982). In cold-treated anthers, the total content of free amino acids is increased, which might be conductive to an adaptation of microspores to the metabolic changes and embryogenesis induction (Krogaard and Andersen 1983, Claparols et al. 1993, Xie et al. 1997). Also, during cold treatment, two small heat-shock protein (HSP) genes, i.e. tom66 and tom111, are expressed possibly to protect cells against chilling injuries (Sabehat et al. 1998). In durum wheat, the cold pretreatment of the whole spike led to an increase in green plant production probably due to the delay of plastid senescence (Labbani et al. 2005; Fig. 3).

Heat shock

Heat shock has been used as a trigger to induce embryogenesis in isolated microspores of rapeseed (Keller and Armstrong 1979, Custers et al. 1994), wheat (Touraev et al. 1996b), tobacco (Touraev et al. 1996a), eggplant (Miyoshi 1996), timothy (Guo and Pulli 2000a) or in cultured anthers of oats (Kiviharju and Pehu 1998), flax (Chen et al. 1998) and *Hepatica nobilis* (Nomizu et al. 2004).

Heat shock has been shown to cause changes in microtubule and cytoskeleton in cultured *Brassica* microspores (Hause et al. 1993, Cordewener et al.

Table 2. Protocols with special emphasis to novel and neglected stress treatments on microspore embryogenesis in selected species. UC, uni-cellular; BC, bi-cellular; E, embryo; R, regenerant; C, callus; IMC, isolated microspore culture; AC, anther culture; MCS, multi-cellular structures; ABA, abscisic acid.

Plant	Stress	Protocol	Microspore stage	Result	System	Reference
Tobacco	pH	pH (8–8.5) in T1 med./4–6 days/ 25°C/ microspore suspension	Late UC	E&R	IMC	Barinova et al. (2004)
Snapdragon	pH	pH (8–8.5) in AT3 med./4–6 days/25°C/ microspore suspension	Late UC	MCS	IMC	Barinova et al. (2004)
Tobacco	Lithium	$LiNO_3$ (5 m*M*)/1 day/25°C/microspore suspension	Early BC	MCS	IMC	Zonia and Tupy (1995)
Broccoli	Carrageenan oligosaccharides and/or heat	λ-Carrageenan oligosaccharides (170 n*M* or 34 μ*M*)/30 min/microspore suspension	Late UC-early BC	Symmetrical divisions	IMC	Lemonnier-Le Penhuizic et al. (2001)
		λ-Carrageenan oligosaccharides (170 n*M* or 34 μ*M*) and 32.5°C/ 24 h/microspore suspension	Late UC-early BC	E&R	IMC	
Tobacco	ABA	ABA (0.01 m*M*)/3 days/anthers	Early BC	E&R	AC	Imamura and Harada (1980a)
Tobacco	Reduced atmospheric pressure	Reduced atmospheric pressure (12 mmHg) in dessicator/10, 20 or 60 min/anthers	Early BC	E&R	AC	Imamura and Harada (1980b)
Wheat	Hypertonic	0.8 *M* sucrose/excised anthers	Mid-to-late UC	C&R	AC	Wang et al. (1981)
Tobacco	Centrifugation	Centrifugation (10 000–11 000 *g*) for 30 min/anthers	Late UC	E&R	AC	Tanaka (1973)
Wheat	Inducer chemicals	2-HNA (0.18 m*M*)/25°C/48 h/ microspores	Mid-to-late UC	E&R	IMC	Zheng et al. (2001)
Wheat	Inducer chemicals and heat	2-HNA (0.01%) + 2,4-D (10 mg l^{-1}) + BAP (2 mg l^{-1}) + gibberellic acid (3 mg l^{-1})/33°C/ 69 h/spikes	Mid-to-late UC	E&R	IMC	Liu et al. (2001)
Maize	Inducer chemicals and cold	2-HNA (100 mg l^{-1})/5–10°C/8–14 days/florets	Late UC to early BC	E/C&R	IMC	Zheng et al. (2003)

1994, Simmonds and Keller 1999). In these microspores, the appearance of preprophase bands

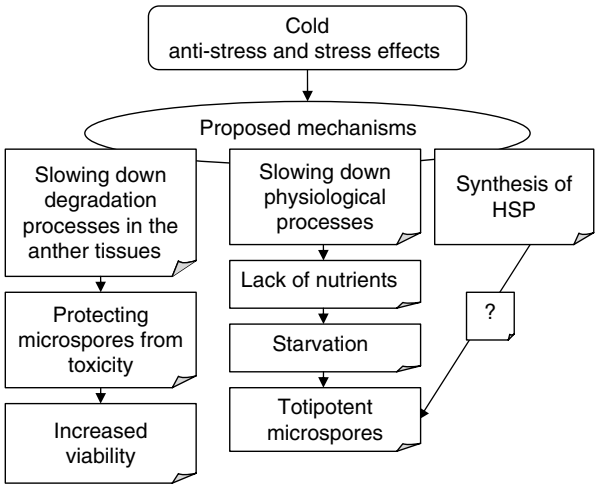

Fig. 3. Cold shock and its proposed mechanisms.

(Simmonds and Keller 1999) was affected and structural changes including electron-dense deposits at the plasma membrane/cell wall interface, vesicle-like structures in the cell walls and organelle-free regions in the cytoplasm were detected (Telmer et al. 1993). As expected, several HSP are synthesized in heat-stressed microspores (Pechan et al. 1991, Cordewener et al. 1995, Binarova et al. 1997, Segui-Simarro et al. 2003) of which HSP70 was suggested to inhibit apoptosis (Jäättelä et al. 1998). In *Brassica* microspores, the heat shock led to endo-reduplication, with G2 haploid nuclei entering in G1 (diploid) without mitosis (Binarova et al. 1993). Furthermore, release of the G1 arrest and entry into S phase of the cell cycle of the vegetative cell take place (Binarova et al. 1997). Also, during heat-induced microspore embryogenesis in *B. napus*, MAP kinases were shown (Segui-Simarro et al. 2005) to be developmentally regulated (for details see Zoriniants et al. 2005; Fig. 4).

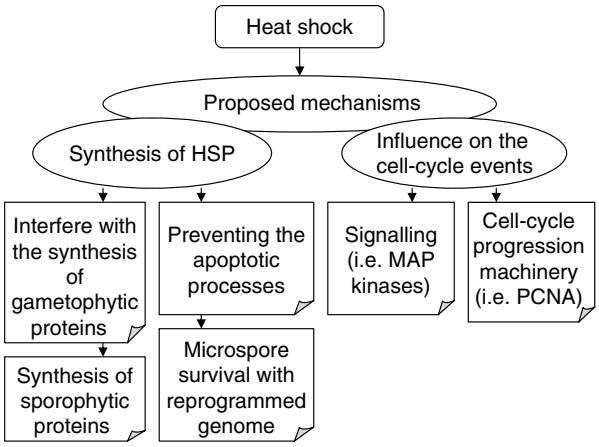

Fig. 4. Heat shock and its proposed mechanisms.

Sugar starvation

Sugar starvation is an effective inducer of embryogenesis in isolated microspores of many important crops such as tobacco (Kyo and Harada 1986, Touraev et al. 1996a), wheat (Touraev et al. 1996b), rice (Ogawa et al. 1994), barley (Hoekstra et al. 1992), apple (Höfer et al. 1999) or in cultured anthers of rice (Raina and Irfan 1998) and rye (Guo and Pulli 2000b, Ma et al. 2004).

Cytoplasmic and nuclear changes have been observed in starved microspores including de-differentiation of plastids, dilation of the generative cell wall, the appearance of a large vacuole, loss of nuclear pores in the vegetative nucleus, changes in chromatin and nuclear structure, phospholipid composition of plasmalemma and a decrease in the size of the nucleolus (Kyo and Harada 1990a, 1990b, Garrido et al. 1995). The decrease of RNA synthesis and protein kinase activities were characteristics of starved tobacco microspores (Zarsky et al. 1990, Garrido et al. 1993). The G1 arrest in the cell cycle of the vegetative cell is de-repressed by starvation (Zarsky et al. 1992). A small HSP gene in starved early bi-cellular pollen grains of tobacco is expressed (Zarsky et al. 1995) which may prevent apoptosis. During the starvation treatment, qualitative and quantitative changes in protein kinase activities were shown (Garrido et al. 1993) (Fig. 5).

Colchicine

Colchicine treatment of isolated *B. napus* microspores with the concentrations 50 and 500 mg l^{-1} for 15 h stimulated embryogenesis and produced large amounts of healthy-looking embryos and high doubling efficiency of 83–91% (Zhou et al. 2002).

Colchicine, a microtubule-depolymerizing agent, disrupts microtubule formation during cell division thus hindering division-related activities of cells completely (Kasha 2005). Microtubules are necessary in the spindle formation for chromosomes to be drawn to the poles, and failure of such an activity may result in endoduplication (Fig. 6). Colchicine was shown to stimulate embryogenesis in isolated *B. napus* (Zaki and Dickinson 1991, Zhao et al. 1996) and coffee (Herrera et al. 2002) microspore cultures, and in maize anther cultures (Obert and Barnabas 2004). The response of microspore to colchicine seems to be dependent on the developmental stage of microspores. In rapeseed, highest frequency of embryogenesis has been obtained in vacuolated microspores, somewhat earlier stage than the population responsive to heat induction (Zhao et al. 1996). Occurrence of the cytoskeletal disruption at a

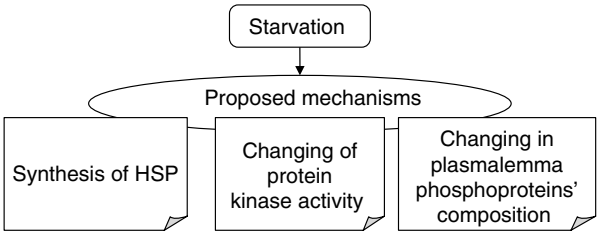

Fig. 5. Carbon starvation and its proposed mechanisms.

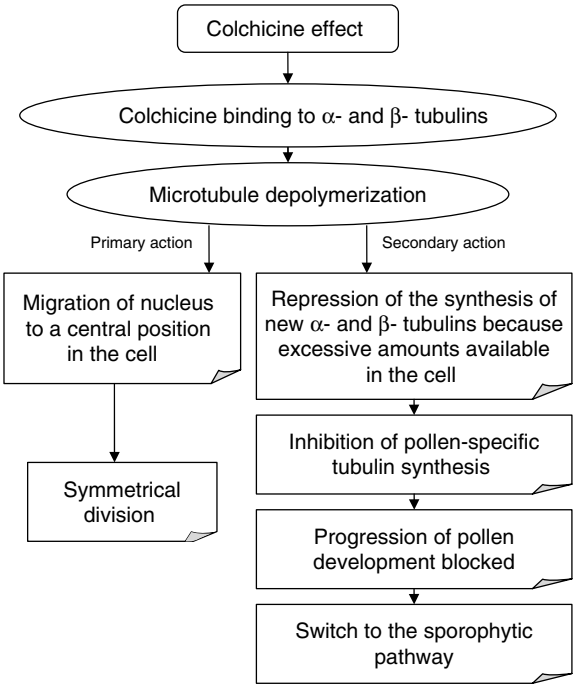

Fig. 6. Colchicine effect and its proposed mechanisms.

particular developmental stage is not surprising, because the microtubules of uni-cellular microspores are much more sensitive to colchicine than those of bi-cellular pollen. Colchicine treatment results in complete depolymerization of uni-cellular microtubules, whereas most bi-cellular microtubules are not affected (Zhao and Simmonds 1995).

Colchicine binds to α- and β-tubulin heterodimers, which inhibits further dimer addition to microtubules and results in eventual microtubule depolymerization (Sternlicht et al. 1983). In the primary action of colchicine during microtubule depolymerization, the nucleus anchored to the cell wall is released, microspore asymmetry is lost (Simmonds 1994), the nucleus migrates to the centre and microspores undergo a symmetrical division (Zhao et al. 1996). These changes in the cell polarity may convert microspores to the sporophytic pathway. Late uni-cellular microspores, entering mitosis during the initial phase of culturing, escape the effects of colchicine and divide asymmetrically, as in the first pollen mitosis, and continue normal gametophytic development (Zhao and Simmonds 1995).

A second action of colchicine, after binding to α- and β-tubulin heterodimers, is that the elevated concentration of free tubulins acts to repress the synthesis of new α- and β-tubulins (Cleveland et al. 1983) which may include the synthesis of pollen-specific tubulins (Carpenter et al. 1992) and thus prevent the progression of pollen development. This effect may contribute in the re-programming of microspores towards sporophytic pathway.

In *B. napus*, heat and colchicine are used in combination to induce microspore embryogenesis (Simmonds 1994). The two appear to act on different microspore developmental stages; however, this is partly because they require different periods of time to exert their effects. Microtubule depolymerization by colchicine is a slow process, requiring up to 8 h (Zhao and Simmonds 1995). Another advantage in using colchicine is that it is also a chromosome-doubling agent. A simple one-step process to simultaneously induce embryogenesis and chromosome doubling for the production of fertile plants is very advantageous for genetic studies and plant breeding programmes.

In wheat, treatment of isolated microspores with colchicine (up to 3 m*M* for 24–48 h) increased the percentage of fertile regenerants from 15% (in untreated control cultures) up to 53% (Hansen and Andersen 1998). In anther, culture of maize colchicine pretreatment (0.02% for 3 days) could initiate embryogenesis (Obert and Barnabas 2004). Also, treatment of isolated coffee microspores with colchicine (100 mg l^{-1} for 48 h) induced embryogenesis (Herrera et al. 2002).

In summary, the efforts on the use of colchicine have been focused on the *Graminaceous* and *Cruciferous* crops in which microspore embryogenesis is already successful. However, reports regarding its effects on recalcitrant species are scarce.

Neglected stresses

γ-Irradiation

Ionizing radiation, e.g. γ-irradiation, first used for the induction of mutants in haploid cells (i.e. microspores), was also applied to increase efficiency of microspore embryogenesis in anther cultures of rapeseed, tomato, *Datura* and *Nicotiana* (Sangwan and Sangwan 1986, Shtereva et al. 1998). γ-Irradiation with the dosage of 10 Gy significantly increased induction of embryogenesis in cultured *Nicotiana* and *Datura* anthers (Sangwan and Sangwan 1986). However, contradictory results have been observed when γ-irradiation was tested in isolated microspore and anther cultures of *B. napus* ssp. *oleifera* Metzg. (Sinsk). Irradiation with 10 Gy significantly increased embryogenic response of cultured anthers, whereas the same or other doses decreased embryogenesis in isolated microspore cultures (MacDonald et al. 1988). Combination of a 4 Gy γ-irradiation followed by incubation at 10°C for 9 days significantly increased anther culture response in tomato (Shtereva et al. 1998).

It was reported that irradiation alters auxin and cytokinin balance in somatic tissues (Klein and Vogel 1956, Degani and Pickholtz 1982). Whether this relates also to microspore embryogenesis remains to be seen.

Ethanol stress

Ethanol has been reported not only as an inducer but also as an inhibitor of microspore embryogenesis. Ethanol stress-induced microspore embryogenesis was reported by Pechan and Keller (1989) in *B. napus* L. However, a high concentration of sucrose (40 m*M* for 6 h) in the culture medium led to increased ethanol production which in turn seemed to kill cultured barley microspores (Scott et al. 1995). Reducing sucrose concentration or replacement with maltose decreased ethanol accumulation and enhanced microspore embryogenesis (Scott et al. 1995). In carrot, it was also shown that unorganized cell growth and somatic embryogenesis in cell cultures were strongly inhibited by ethanol at relatively low concentrations (10–20 m*M*) (Perata and Alpi 1991).

Hypertonic shock

Treatment of excised wheat anthers with 0.8 *M* sucrose prior to culture was shown to increase the induction frequency of microspore-derived calli (Wang et al. 1981). In another report, excised wheat anthers were pretreated with 0.8 *M* sucrose and activated charcoal (3%) for 1–2 h prior to culture in several spring and winter genotypes (Zhang et al. 1987). There was no effect of pretreatment with spring genotypes. However, a favourable effect was obtained with winter genotypes, which showed an increase of the induction of both microspore-derived calli and regenerated green plants (Zhang et al. 1987). More recently, Supena et al. (2005) reported that prefeeding of pepper (*Capsicum annuum* L.)-isolated microspores in rich medium containing 6% sucrose for 4 h before transferring to the sugar-free B medium containing mannitol allowed sporophytic divisions to start during the starvation period and increased frequency of embryogenic microspores up to 15%. It can be considered that using high osmolarity of metabolizable carbohydrates for short time to anthers or isolated microspores before applying stress may help them divide during stress treatment and tolerate stress conditions.

Centrifugal treatment

The effect of centrifugal shock on the emergence of plantlets from cultured anthers of tobacco was reported by Tanaka (1973). In this experiment, tobacco anthers at the late uni-cellular stage as well as at the early bi-cellular stage were subjected to a centrifugal force corresponding to 10 000–11 000 *g* for 30 min. Approximately four-fold increase of regeneration of haploid plants were observed. However, the treatment of anthers at the early bi-cellular stage had little influence (Tanaka 1973). It was concluded that the centrifugal treatment facilitates the differentiation of embryos from microspores. This approach of pretreatment can be tested further in isolated microspore cultures.

Reduced atmospheric pressure

Reduced atmospheric pressure, when applied to tobacco anthers, was shown to stimulate embryogenesis from bi-cellular pollen (Imamura and Harada 1980b). After 10 min pretreatment at 12 mmHg, almost 64% cultured anthers produced plantlets whereas the control remained at 6.1%. Average number of plantlets per anther was 10 times higher at 20-min treatment compared with the control (17.3 vs. 1.7). It was proposed that reduced level of atmospheric O_2 or the rapid exhaustion of inhibitory gases produced by the anthers during the initial phase of culture may favour the microspore embryogenesis.

Feminizing agents

The enhancing effect of naphthalene acetic acid and Alar 85 as feminizing agents and nitrogen starvation as a feminizing factor on the frequency of sterile and embryogenic pollen grains (P-grains) *in planta* in *N. tabacum* was observed when applied to the donor plants via spraying and soil drenching (Heberle-Bors 1983). However, in the same report, Ethrel, Cycocel and GA_3, known to affect sex determination, were ineffective, when applied in a similar manner. Ethrel, which induced additional mitosis in wheat pollen (Bennet and Hughes 1972), was reported to be ineffective in anther culture of *C. annuum* (MacDonald and Grant 1974) but effective to enhance anther-derived plants in rice (Hu et al. 1978). Further tests on the use of above compounds may prove their usefulness for the induction of microspore embryogenesis.

ABA

The stimulatory effect of ABA (0.01 m*M*) on microspore embryogenesis was shown in 3-day-pretreated tobacco anthers (Imamura and Harada 1980a). Moreover, in *Hordeum vulgare* L., addition of ABA to anther cultures increased the viability and lowered the occurrence of apoptotic characteristics in the microspores (Wang et al. 1999). Because ABA acts as an inhibitor of mRNA synthesis, it may be possible that treatment with ABA inhibits synthesis of certain RNAs necessary for gametophytic development thereby blocking gametophytic pathway and thus switching on the sporophytic development (Imamura and Harada 1980a). Pretreatment of anthers with ABA stimulated microspore viability and regeneration efficiency in barley (Van Bergen et al. 1999). It was determined that in starved barley and tobacco microspores, endogenous ABA levels were several fold higher during the initiation of the culture (Imamura and Harada 1980a, Van Bergen et al. 1999). It appears that the ABA is effective in preventing the death of microspores and repressing further development of microspores into maturity (Wang et al. 2000). It can be speculated that the starvation pretreatment may be replaced by ABA pretreatment in species which cannot withstand long carbon starvation pretreatments.

Novel stresses

High medium pH

Manipulation of medium pH in the induction of microspore embryogenesis is a novel approach (Barinova et al. 2004). Tobacco and snapdragon microspores cultured in T1 and AT3 media, respectively, at pH 7 developed to the normal pollen grains and germinated readily in germination medium. However, following initial incubation of tobacco and snapdragon microspores in T1 and AT3 media, respectively, at pH 8.0–8.5 for 4–6 days, symmetrical division of the microspore nucleus occurred at significantly high frequencies producing two large nuclei of equal size. Following the initial incubation at high pH upon transfer to AT3 medium with a pH of 6.5, multicellular structures were obtained in both tobacco and snapdragon. In tobacco, formation of embryos followed and after germination plants were grown to maturity. The authors also studied the mechanism of the high pH effect and determined that invertase activity strongly decreased at higher pH values resulting in slowing down of sucrose cleavage, the maximum cleavage being at pH 5.0 as determined by invertase activity. Sucrose uptake in tobacco microspores was reduced in high pH determined by the level of ^{14}C-labelled sucrose. The authors concluded that the microspores starve at high pH due to reduction in sucrose uptake as well as disruption of cleavage of sucrose. Such a starvation following re-programming leads to sporophytic development.

Carrageenan oligosaccharides

Oligosaccharide elicitors, also known as oligosaccharidic degradation products or oligosaccharins, from the cell walls of higher plants, fungi or bacteria were also shown to induce microspore embryogenesis (Lemonnier-Le Penhuizic et al. 2001). In addition to physical stresses, the idea of using molecules mimicking biotic stresses in the induction of microspore embryogenesis is very attractive. A total of eight molecules, namely κ-carrageenan, ι-carrageenan, λ-carrageenan, agar, laminarin, alginate, fucan and pectin, were tested for their effect on the induction of embryogenesis in cultured microspores of *Brassica oleracea* var. italica (Lemonnier-Le Penhuizic et al. 2001). Only carrageenan oligomers, i.e. κ-carrageenan, ι-carrageenan and λ-carrageenan, were found to be effective in the induction of microspore embryogenesis. A pretreatment with λ-carrageenan, in combination with the heat shock at 32.5°C for 24 h, led 80% of cultured *Brassica* microspores to divide symmetrically, and the embryo yield increased two-fold (Lemonnier-Le Penhuizic et al. 2001). Treatments with carrageenan oligomers as short as 30 min were effective, although continuous presence of carrageenan oligomers in culture media did not decrease the efficiency of embryogenesis. The optimum concentrations of ι-carrageenan oligosaccharides were determined to be in the range of 170 n*M*–34 μ*M*. Interestingly, embryo-derived plantlets formed approximately 1–2 weeks earlier in media containing carrageenan oligosaccharides compared with the control cultures. It was suggested that the carrageenan oligosaccharides act as signal molecules enhancing the induction of microspore embryogenesis in *B. oleracea* var. *italica* but did not have an effect for embryo conversion. It is, however, too early to propose a wider use of the carrageenan oligosaccharides in other species.

Heavy metal stress

Zonia and Tupy (1995) reported that the normal developmental pathway of *N. tabacum* microspores can be blocked or switched when microspores are exposed to lithium, and these effects are reversible with Ca^{2+} and myo-inositol. An asymmetrical partitioning of membrane-associated Ca^{2+} occurs during microspore development within the anther (Zonia and Tupy 1995). Moreover, an asymmetrical distribution of membrane-associated Ca^{2+} in microspores cultured in vitro was observed. However, microspores cultured in the presence of lithium did not have asymmetrical distribution of membrane-associated Ca^{2+} suggesting that lithium blocks the normal asymmetrical partitioning of membrane-associated Ca^{2+}, possibly by interfering with some regulators of Ca^{2+} partitioning or Ca^{2+} mobilization (Zonia and Tupy 1995). Approximately, a four-fold increase in the frequency of symmetrical mitosis was obtained via lithium treatment. However, symmetrically divided microspores could not develop into embryos and only formed four- or five-celled proembryo-like structures (Zonia and Tupy 1995). The inhibitory effect of lithium on inositol 1-phosphatase has already been reported (Jackson et al. 1989, Gani et al. 1993). Lithium shuts down inositol phosphate signalling by inhibiting inositol-1-phosphatase (Jackson et al. 1989, Gani et al. 1993) and preventing the re-supply of free myo-inositol (Berridge et al. 1989) that is required for signal propagation. Inhibition of inositol phosphatase cycling also blocks the activation of intracellular Ca^{2+} pools and interferes with the establishment of cellular polarity (Busa and Gimlich 1989) resulting in an alteration in the mitotic asymmetry and formation of two equal nuclei, which is an important phenomenon in microspore embryogenesis induced by stress and may be required

to switch the developmental pathway from gametophytic to sporophytic. Heavy metals, such as CdCl at 0.6 *M* concentration, as a stress treatment in the induction of somatic embryogenesis have been successfully used previously (Kiyosue et al. 1990, Ikeda-Iwai et al. 2003).

Inducer chemicals

Several chemicals, such as 2-hydroxynicotinic acid (2-HNA), anthranilic acid (AA), benzotriazole (BT), BT-5-carboxylic acid (B-5-CA), 2,3-butanedione monoxime (2,3-BM), DL-histidine (DL-H), 2,4-dihydroxy pyrimidine 5-carboxylic acid (2,4-DPCA), 2,3-pyridine carboxylic acid (2,3-PCA), sulfanilamide and violuric acid monohydrate (VAM), were tested for their effect in the induction of microspore embryogenesis and/or enhancement of regeneration frequency and percentage of green plants in wheat (Liu et al. 2001, Zheng et al. 2001). It was determined that all compounds, except 2,4-DPCA, increased viability of microspores significantly compared with the control. Among them, 2-HNA, B-5-CA and VAM were also effective in enhancing the frequency of embryogenic microspores substantially (Zheng et al. 2001). Because developmental pattern of microspores treated with B-5-CA and VAM was almost identical to those treated with 2-HNA, in further studies only 2-HNA was used. It was observed that the use of 2-HNA has the positive effect on embryo yield, percentage of green plants and the frequency of spontaneously doubled haploids for all the genotypes tested (Zheng et al. 2001).

It was concluded that making some nutrients available to microspores pretreated with inducer chemicals at the stage when embryogenesis is triggered is an important factor affecting plant regeneration and green plant production (Liu et al. 2001). The inducer chemicals may be useful in the induction of embryogenesis in other species as well.

2,4-D pretreatment

Both as a synthetic auxin and a herbicide, 2,4-D has found an extensive use in tissue culture systems (Gaj 2004). Not only is it used for its growth regulator effects but also as a stress treatment especially when used at high concentrations (Dudits et al. 1991, Gallo-Meagher and Gerrn 2002). Also, isolated and unfertilized egg cells of maize were induced to develop sporophytically following pretreatment with 2,4-D at high concentrations, i.e. 25, 30 and 40 mg l^{-1} for 1 h (Kranz et al. 1995). Regulatory effect of 2,4-D on endogenous auxin metabolism, namely IAA metabolism, which plays significant role in somatic embryogenesis, was reported (Michalczuk et al. 1992a, 1992b). However, in microspore embryogenesis, reports are not available on the use of 2,4-D as a stress pretreatment at high concentrations. Moreover, experiments performed by the authors' group on the effect of high concentration of 2,4-D, i.e. 20–100 and 200 mg l^{-1} for hours to 1 day at 25°C in tobacco confirmed that use of 2,4-D alone is not sufficient to induce embryogenic microspores but increased significantly the frequency of symmetrically divided bi- and three-cellular structures (unpublished). Perhaps 2,4-D can be used in combination with other stresses to increase sporophytic divisions.

Conclusions

From the foregoing discussion, it can be concluded that firstly, the choice of stress will depend on its efficiency in the formation and frequency of embryogenic microspores, overall viability, functionality and practicality. If, for example, cold treatment was as efficient as colchicine or γ-irradiation pretreatments, the classical cold treatment will be preferred because the others will require extra handling and technical means. Moreover, to minimize the side effects of stresses, one should consider applying them as mild as possible.

A second important conclusion is that we still do not know exactly what feature of the microspores can be introduced as universal sign of the embryogenic (totipotent) state. Whereas 'star-like' microspores seem to be embryogenic in wheat, tobacco, apple and rice, embryogenic *B. napus* microspores do not have a star-like cytoplasmic structure but show an increase in the size of the cell and symmetrical divisions. However, it was shown that depending upon the type of stress used both symmetric and asymmetric first cell divisions are found in embryogenic barley microspores (Hu and Kasha 1999). The first division in embryogenic wheat microspores is usually symmetric when mannitol pretreatment is used, whereas it is asymmetric after cold pretreatment (Hu and Kasha 1999).

A third conclusion is that we are far from understanding precisely what stress means in the context of microspore re-programming. While heat and starvation treatments share certain features in their effect on microspore embryogenesis in a wide range of different species, cold cannot really be considered a stress, but rather an antistress. A consistent feature in all the investigations on cold stress was that it maintained viability during re-programming while the trigger seemed to be starvation. For colchicine and the majority of neglected and novel stresses, we lack evidence for their general relevance in microspore embryogenesis and for their stress effect. We also do not know whether or when the different stresses converge on a common triggering

pathway in the conversion of a gametophytic microspore to a sporophytic one. Although HSP seem to be induced after treatment of microspores with a number of different stresses, it is still not known whether they play a direct role in re-programming.

Another issue is whether the stress is really triggering or conditioning the microspores for embryogenesis. In the former case, stress is considered the primary agent of change, whereas in the latter case, stress is seen as a secondary factor supporting, as in other tissue culture systems, the role of hormones. Often overlooked, it has to be emphasized that the culture media in most microspore culture systems do not contain hormones, whereas in those few species where hormones are employed at the start of culture, they are used at high concentrations, actually acting like a stress. Similarly, in some somatic embryogenesis systems, such as alfalfa, 2,4-D acts as a stress (Pasternak et al. 2002). Still, in some other microspore culture systems, hormones are added after the stress treatment such as in wheat where they seem to play the role of conditioning factors in the sense that they improve embryo quality.

Thus, it seems that stress is indeed the decisive trigger diverting microspores from their normal development. Without stress, microspores will invariantly develop into pollen grains. Whether microspores can be called stem cells, similar to cells in meristems, is difficult to tell. Microspores are surrounded by a highly specialized cell wall, the exine, but do not show specialized features inside the cell. These are formed only later, during pollen development and maturation. Their ability to become totipotent upon stress treatment should not be used as an argument that microspores may be called stem cells because other differentiated plant cells can also be induced to become totipotent.

Some stresses can be used in a wider range of species, whereas others seem to be applicable only in a restricted number. For example, heat and starvation alone or in combination are effective in tobacco (*Solanaceae*), wheat (*Poaceae*) and *B. napus* (*Brassicaceae*). However, in tomato, which is in the same family as tobacco, those stresses do not work. Moreover, whereas in *B. napus* microspore, embryogenesis can be induced easily by heat shock, in its closely related species, *Arabidopsis*, there exist no microspore-derived embryos. In attempts to produce doubled haploids in a new species, it may thus be advisable to try first the established stresses and then turn to the novel ones. The neglected stresses may be neglected for a reason, i.e. they may have been tried but failed to be reproducible. However, because many such failed trials may not have been published, it is difficult to tell whether they are really ineffective.

The last issue is gene expression during the induction of microspore embryogenesis. Genes specifically expressed during embryogenic induction of microspores have been studied extensively (Maraschin et al. 2005). Although some genes have been reported to be upregulated during microspore embryogenesis, so far, no single gene has been characterized to be really specific for embryogenic microspores or essential or even necessary for microspore re-programming. Thus, we still lack a coherent description of the process of microspore re-programming on the molecular level.

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