Direct somatic embryogenesis and organogenesis pathway of plant regeneration can seldom occur simultaneously within the same explant of sorghum

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Introduction

Sorghum (*Sorghum bicolor*) is an important staple food crop, especially of the poor in Africa, Asia and Central America and needs to be improved through genetic engineering. Generation of transgenic plants depends mainly on the standardization of an efficient tissue culture and regeneration protocol. Sorghum tissue culture and regeneration protocols mostly follow either organogenesis (Maheswari et al. 2006) or direct somatic embryogenesis pathway (Harshavardhan et al. 2002). Following the protocol of Girijashankar et al. (2005), we attempted to produce transgenic sorghum with synthetic *Bt* gene constructs (*ubicry1Ab* and *ubicry1Ac*, separately). However, molecular analysis of the T_0 and their progeny plants revealed the loss of germline transmission of the respective transgenes under study. Recent studies by Wang et al. (2005) with *Agrobacterium*-mediated transformation of maize (*Zea mays*) and regeneration through embryogenic callus, reported the absence of transgenes in the progeny plants. We report here the occurrence of different types of calli, few of which are not expected to occur during the direct somatic embryogenesis pathway of sorghum regeneration protocol followed to obtain the above transgenic plants.

Materials and methods

Plant material. Sorghum genotype BTx623, a popular seed parent, was used in this study. Shoot apices of 7-day-old seedlings were excised and placed horizontally on somatic embryo stimulation medium (SESM) (a cytokinin rich medium), containing the Murashige and Skoog (MS) basal medium fortified with 5 mM of thidiazuron (TDZ), 4.0 mg L^{-1} of benzylaminopurine (BAP) and 0.1 mg L^{-1} of naphthalene acetic acid (NAA) and bombarded.

Plant regeneration. The bombarded explants were incubated for 15 days on SESM. These explants were further sub-cultured (for a fortnight) onto a somatic embryo induction medium (SEIM) containing MS supplemented with 4 mg L^{-1} of BAP and 0.5 mg L^{-1} of 2,4-dichlorophenoxy acetic acid (2,4-D) (a strong auxin). Later, the meristematic masses were transferred onto somatic embryo germination medium (SEGM) composed of MS, 4 mg L^{-1} BAP and 0.1 mg L^{-1} NAA, where the somatic embryos germinated and developed into well-differentiated shoot apex surrounded by a pair of primary leaves. After a fortnight these were sub-cultured onto shoot elongation medium (SEM) comprising MS fortified with 1 mg L^{-1} BAP and 0.5 mg L^{-1} indolebutyric acid (IBA) and sub-cultured fortnightly. The remaining process of rooting, hardening and acclimatization were according to Girijashankar et al. (2005). Thus, the regeneration of bombarded explants was carried out by direct somatic embryogenesis over a period of 16 wk.

Results and discussion

Development of callus was not observed in any of the explants (shoot apices on SESM) during the first passage. However, formation of soft, white, non-embryogenic callus was observed initially on the explants in SEIM. This type of callus mostly occurred at the margins of the meristematic shoot clumps from which multiple direct somatic embryos (DSEs) were developing (Fig. 1a). Further, this callus continued to occur randomly at various stages during in vitro regeneration. The interesting and unique aspect is the formation of yellow, globular, compact embryogenic callus which got initiated when the explants were sub-cultured onto SEIM (Figs. 1b–d) that contained 2,4-D (0.5 mg L^{-1}). However, it is to be noted that only few meristematic clumps developed callus at one or the other stage of regeneration (31/346 explants), which is attributed to the use of the cytokinins namely TDZ and BAP in the first stage of incubation in SESM.

Incredibly, it is observed that DSEs were not affected by the callus formation because they grew fast and produced a pair of expanding primary leaves from the

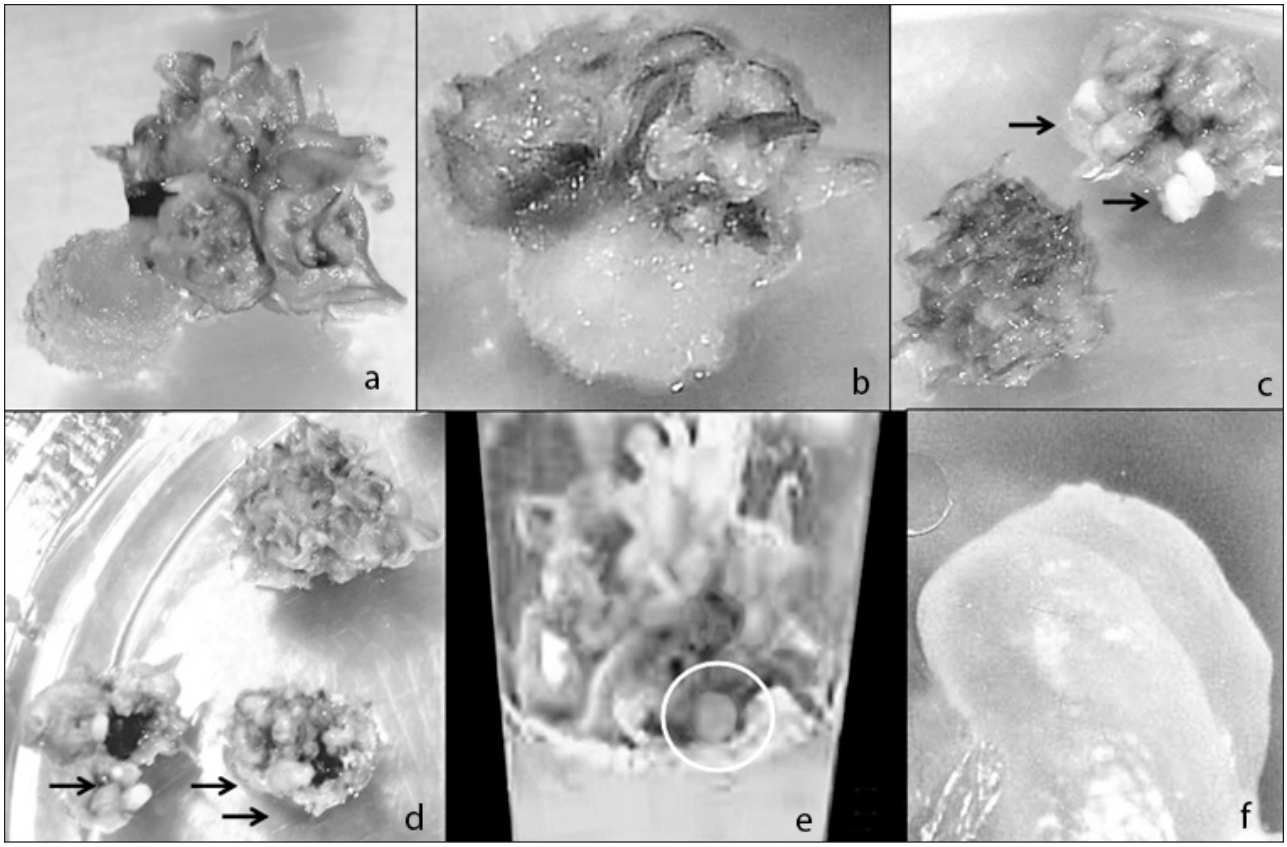

Figure 1. Occurrence of non-embryogenic as well as embryogenic callus during in vitro plant regeneration from sorghum shoot apices: (a) Formation of whitish friable non-embryogenic callus on the same explant showing the development of multiple somatic embryos; (b) Occurrence of yellow compact and globular embryogenic callus in somatic embryo induction medium; (c, d) Development of direct somatic embryos (DSEs) and organogenic embryos on the same medium side by side in the same explant. Arrows show yellow granular calli which are capable of producing embryos. Adjacent to these explants are other explants developing multiple DSEs without any callus phase in the same petri plate; (e) Compact yellow germinating somatic embryo in between the developing shoots in shoot elongation medium; (f) Organogenic embryo generated from callus mass clearly showing a bifurcation patch.

somatic embryos in the SEGM. The compact, yellow and globular embryogenic calli capable of regenerating into plantlets are also seen growing adjacent to the developing shoots originated from DSE in SEM (Fig. 1e); embryogenic as well as non-embryogenic calli continued to occur in the SEM. These yellow nodular embryogenic calli that developed in between the DSEs could not be eliminated because of their small and compact nature which made them difficult to handle. Further, removal of these yellowish embryos leads to the breakage of explants into too small fragments rendering them unable to regenerate efficiently. Among the yellow, globular calli that were observed, only few of them continued to enlarge in the SEGM and eventually produced a heart-shaped bifurcated somatic embryo as protuberance from the callus clump in SEM (Fig. 1f). On the same meristematic shoot clump, by the time the callus produced embryos, the adjacent DSEs developed into shoots with a minimum of two small leaves. On the other hand, the white and soft callus continued to remain as cell mass but never developed into embryos.

Thus, 91% (315/346 explants) produced only multiple DSEs, while 9% (31 explants) of the apical meristems developed callus in between the DSEs within the same explants and nutrient medium. The mechanism of mixed growth of both DSEs and organogenic embryos originating and maturing in a single explant of sorghum is far from clear. However, the role of 2,4-D (0.5 mg L^{-1}), used in the SEIM (during second passage), cannot be ruled out as the bulged meristematic masses developed callus by the end of 15 days incubation in this medium only. Seetharama et al. (2000) indicated the role of 2,4-D

in induction of callus in sorghum shoot apical meristems. Harshavardhan et al. (2002) reported that MS medium supplemented with BAP (2–4 mg L^{-1}) and 2,4-D (0.5 mg L^{-1}) was always accompanied with certain degree of callus formation and replacement of 2,4-D with NAA resulted in the effective induction of somatic embryos without any callus formation. The callus formation observed in our experiment is in accordance with Harshavardhan et al. (2002). We report here for the first time and also provide evidence for the occurrence of this unique phenomenon of sorghum plant regeneration from shoot apical meristems. The protocol followed here needs to be fine tuned so as to eliminate the callus occurrence.

The white and soft calli are non-embryogenic in nature, while few yellow and globular calli produced bifurcated somatic embryos which protruded out from the callus. The two major possible reasons for the loss of germline transmission of transgenes could be either (i) deletion of the transgenes by the host genome by self-DNA protection system (Iyer et al. 2000, Wang et al. 2005); or (ii) regeneration of sorghum plants through intervening callus phase.

These results show the occurrence of both DSEs as well as two different types of callus developing parallelly on the same explant. The occurrence and development of different calli generally goes unnoticed in the laboratory conditions and mostly eliminated during sub-culturing phase. It is not known whether these organogenic embryos regenerated into complete plantlets. These stages throw light on the occurrence of two different plant regeneration pathways on the same sorghum explant.

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