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Storage Protein Patterns in Somatic and Zygotic Embryos of Lentil (*Lens culinaris* Medik.)

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Abstract

In a program aimed at developing a reproducible transformation-capable-regeneration system for lentil only somatic embryo-like bodies (SEB) were obtained. In order to find out whether SEBs are true somatic embryos, storage protein patterns in somatic and zygotic lentil embryos were compared by SDS-PAGE and Western blot analysis. As control, proteins from lentil leaves as well as zygotic embryos were investigated. As no lentil-specific antibodies were available, four different polyclonal antibodies against each of the major storage protein families in pea (legumin, vicilin, phaseolin and glycinin) were applied. The storage protein patterns found in zygotic embryos were also found to occur in somatic embryos. No specific signals were observed in leaf extracts.

Introduction

Knowledge about the synthesis of storage proteins and their accumulation in tissue culture derived somatic embryos of crop plants is limited. Crouch (1982) reported accumulation of storage protein in non-zygotic *Brassica* embryos while Stuart et al. (1985) not only discovered its presence in the somatic embryos of *Medicago sativa* but used it as a criterion to measure embryo quality. In cotton, storage protein deposition in somatic embryos in principle paralleled the processes described for zygotic embryos (Shoemaker et al. 1987).

In contrast to somatic pea embryos derived from immature zygotic embryos or shoot apex (Kysely et al. 1987; Kysely and Jacobsen 1990), those regenerated from protoplast derived calli did not show fully expanded cotyledons (Lehminger-Mertens and Jacobsen 1989).

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In the present investigation it has been possible to develop a large number of somatic embryo-like-bodies (SEBs) from decapitated embryo explants of lentil (*Lens culinaris*) initially cultured on MSB medium containing TDZ (5 - 10 µM) and later subculturing them on MSB medium supplemented with different concentrations of BAP, Kn and NAA. The above SEBs did not grow into fully developed plants. Failure of SEBs to undergo the normal process of development prompted us to study whether or not typical storage proteins (in particular legumin, vicilin, phaseolin and glycinin) are synthesized in SEBs as compared to the formation of zygotic embryos and the differences in the patterns of development of storage proteins in case of their synthesis in SEBs.

Materials and Methods

Plant materials and in vitro culture : Three different genotypes of *Lens culinaris* Medik. were used for *in vitro* plantlet development through organo- and embryogenic means from different explants. For the analysis of storage protein synthesis in somatic embryos, decapitated mature zygotic embryos were cultured on MSB (macro- and micro nutrients of MS (Murashige and Skoog 1962) and vitamins of B5 (Gamborg et al. 1968) supplemented with 10 µM TDZ for four weeks and subcultured on MSB medium containing various concentrations of BAP, Kn and NAA, followed by one more subculture on hormone free MSB medium for one week. Embryos up to the globular stage were harvested from the embryogenic callus and used for protein extraction. Proteins of zygotic embryos and youngest leaves were extracted from the greenhouse-grown lentil plants to serve as control.

Protein extraction : 150 mg of somatic- and zygotic embryos and leaf tissue were homogenized in an electrical homogenizer with 300 µl of extraction buffer I or II (Table 1). All extracts were centrifuged at 15000 ∇ g for 15 min at 4_C. After homogenization 150 µl of supernatants were taken into the fresh tubes.

Gel electrophoresis : Equal volumes of 2 ∇ SDS - sample buffer were added to each tube and placed on a water bath at 80_C for about seven min for denaturation. The protein content was determined according to Esen (1978). Comparable amounts of protein were separated on a 12% SDS slab gel with a 5 % stacking gel (Murray and Key 1978). The gels (8 ∇ 10 cm) were run for 2 h at 12 mA at room temperature in a Biometra-apparatus. Molecular weight standards (Sigma) were run with each gel. The gels were either stained with Coomassie brilliant blue 250 and de-stained with 10 % acetic acid or blotted to nitrocellulose.

Western blot and immunostain : Western blotting (Towbin et al. 1979) was performed in a semi-dry blotting chamber (Biorad); the sandwich was composed according to the manual. The blots were run for 45 min at room temperature with 5.5 mA/cm^2 of nitrocellulose. After blotting, the nitrocellulose was stained with Ponceau for controlling correct transmission of proteins, destained with water and stored in the blocking solution at 4_C until immunostaining (Pratt 1984). Four sets of polyclonal antibodies raised against the mature storage proteins were used for immunostaining.

Table 1. Extraction buffers.

Salt	Buffer I (mM)[1]	Buffer II (mM)[2]
KCl	14.9	
KH_2PO_4	27.2	
$MgCl_2$	9.5	
NaCl	468.3	500
Na_2HPO_4	204.7	32.2
$NaH_2H_2PO_4$		17.8
PVP 44000	2.0	
Tween-20	0.05	
pH 7.0		

[1]According to Domoney et al. (1980), [2]Gaul (1988).

Results and Discussion

In vitro somatic embryo development : In the present investigation it has been possible to induce embyogenic calli from the epicotyl and decapitated mature zygotic embryos initially cultured on MSB medium containing 5 - 10 µM TDZ (Fig. 1A). These embryogenic calli upon subculture on MSB medium supplemented with 0.5 mg/l each of BAP and Kn and 0.2 mg/l NAA produced numerous new embryods with different shapes and sizes (Figs. 1B, C). The embryogenic calli were cultured on hormone free MSB medium before subculturing on the same medium supplemented with BAP, Kn and NAA. However, the newly developed embryods did not germinate to produce fully developed plants.

SDS-PAGE : In order to find out whether or not the SEBs really resulted somatic embryos, the polypeptides of somatic- and zygotic embryos and the leaf tissues were separated on a SDS-PAGE (Fig. 2). Polypeptide patterns of somatic- and zygotic embryos and leaf tissue differed considerably in their

composition i.e., in the number and intensity of the bands. As against seven distinct bands in the zygotic embryo profile, somatic embryos showed 12 distinct bands and some faint bands. Zygotic and somatic embryos shared only four common bands (mol. wt. 29, 36, 40 and 66 kDa). The common bands were conspicuously absent in case of leaf tissues which served as control (Fig. 2).

It may be mentioned that irrespective of tissues used from somatic/ zygotic or leaf for extraction of proteins there was no obvious differences in banding patterns between the two buffer systems.

Immunostain : Western blotting was done to find out the difference, if any, in the banding patterns among the leaf tissue, zygotic- and somatic embryos. After immunostaining with α-glycinin considerable variation in number and intensity of banding patterns was observed in the above samples (Fig. 3).

We observed 12 prominent and four faint bands in the zygotic embryo profile as against five prominent bands in somatic embryos. At least three bands (mol. wt. 40, 45 and 68 kDa) were common between the zygotic- and somatic embryos. These common bands were not present in the leaf tissues.

As has been already mentioned that lentil is considered to be one of the most important sources of dietary proteins for common people of most of the developing countries. However, due to several biotic and abiotic stress factors, the productivity of this crop is hampered considerably. Various attempts have been made in the past to develop disease resistant lines through conventional breeding as well as through biotechnological methods (Williams and McHughen 1986; Saxena and King1987; Polanco et al. 1988; Singh and Raghuvanshi 1989; Malik and Saxena 1992; Warkentin and McHughen 1992,1993; Polanco and Ruiz 2001; Öktem et al. 1999). However, the available literatures indicate that until now there is no efficient routine protocol for *in vitro* regeneration or genetic transformation either through *Agrobacterium*-mediated or through particle gun treatment. In the present investigation we have attempted to develop *in vitro* plant development in lentil through organogenic as well as through embryogenic methods, commonly applied in any standard transformation experiment.

Although there are some reports on the *in vitro* shoot regeneration using cotyledonary nodal explants (Singh and Raghuvanshi 1989; Warkentin and McHughen 1993; Öktem et al. 1999; Gulati et al. 2001; Fratini and Riuz 2002), our experience on transformation experiments shows that the cotyledonary explants are not readily amenable to *Agrobacterium*–mediated transformation. This background information led us choose the decapitated embryo explants,

where the transient GUS expression was maximum. Although in the present investigation it has been possible to induce embryogenic callus from decapitated embryos as well as epicotyl explants on MS/MSB media containing 5 - 10 µM TDZ the embryoids did neither develop further nor show any sign of premature germination. This prompted us to investigate as to whether the developed SEBs were in fact somatic embryos or some other embryo-related structures.

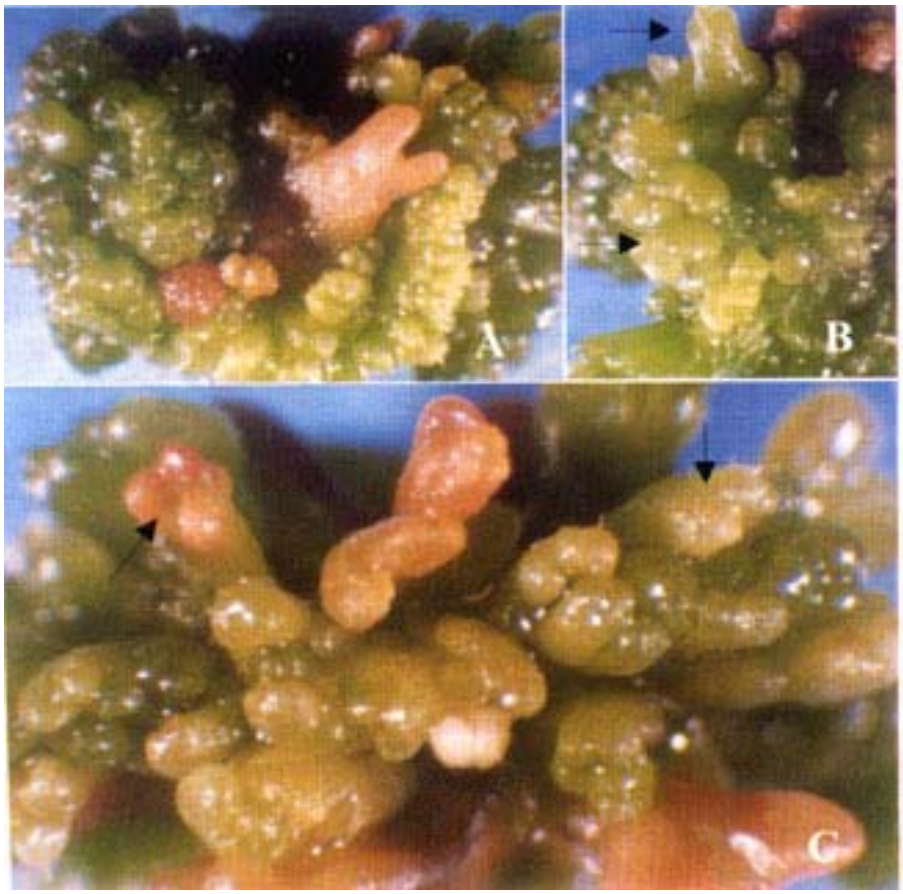

Fig. 1. *In vitro* somatic embryo (SE) development from decapitated embryo explants of lentil on MSB + 10 µM TDZ (A) and on MSB medium supplemented with 0.5 mg/l BAP + 0.5 mg/l Kn + 0.2 mg/l NAA (B,C); arrows indicate the SE of different shapes and sizes.

The results of SDS-PAGE and immunostaining showed that some bands are common between the zygotic embryos and the *in vitro* derived callus lines, thereby indicating that α-glycinin can recognize the polypeptides in both zygotic- as well as in the somatic embryos. This kind of investigation was also

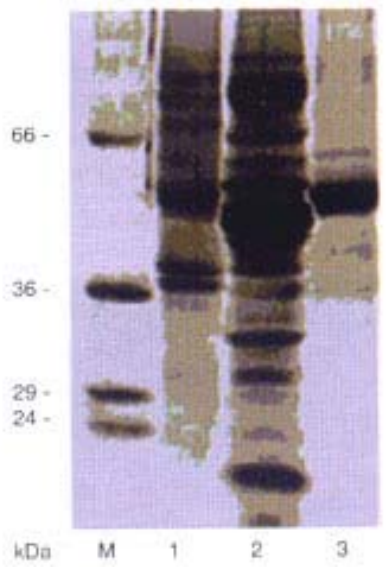

Fig. 2. SDS - PAGE : Lane 1 - zygotic embryo, lane 2 - somatic embryo and lane 3 - leaf tissue.

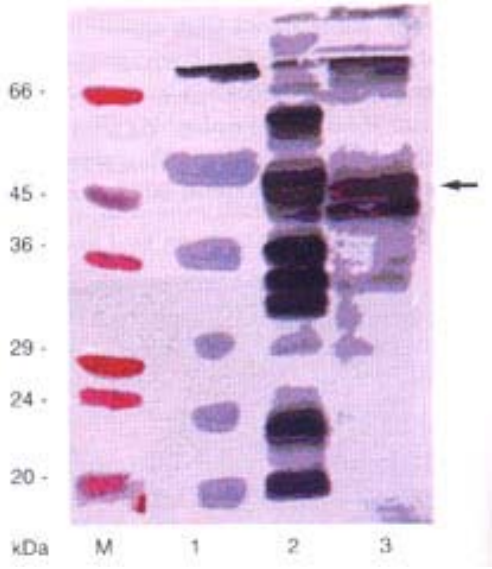

Fig. 3. Immunostain with α- glycinin : Lane 1 - leaf tissue, lane 2 - zygotic embryo and lane 3 - somatic embryo.

made by some other workers for evaluation of genetically engineered rice with soybean glycinin (Momma et al. 1999) and also to find out the properties of tofu and soy milk prepared from soybeans containing different subunits of glycinin (Tezuka et al. 2000). From the foregoing discussion it may be concluded that although it has been possible to induce somatic embryos in our experimental material, the protocol for their maturation and germination needs to be standardized and then can be conveniently used for transformation of lentil mediated either by *Agrobacterium* or particle gun bombardment.

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