Peroxidase as a developmental marker in plant tissue culture

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ABSTRACT Peroxidase was studied as a developmental marker in pumpkin (*Cucurbita pepo* L.) callus lines and horse-radish *(Armoracia lapathifolia Gilib)* transformants. Embryogenic callus lines DE grown on MS medium with 2.4-D and NA-3 grown on medium with NAA and adenine sulfate showed about a 20 times higher enzyme activity than the habituated non-embryogenic line Z5b/T grown on medium without hormones. A rise in peroxidase activity indicated that somatic embryogenesis was triggered in a few habituated tissue cultures. Separated globular embryoids had a manifold lower enzyme activity than the callus from which they originated. SDS-electrophoresis showed distinct polypeptide patterns between the horse-radish leaves and crown galls, but the tumor characteristic protein bands failed to be identified. In horse-radish crown galls and short bushy plants regenerated from hairy roots an enhanced peroxidase activity was registered. Due to its high peroxidase level and abundant biomass production horse-radish transformants should facilitate enzyme production.

KEY WORDS: *pumpkin, horse-radish, peroxidase, callus lines, transformants*

Introduction

Considering that all the cells which build a multicellular organism contain identical genetic information, the problem of cell differentiation remains open in both animal and plant organisms.

Plant cells are characterized by high plasticity in their developmental patterns showing even the ability to regenerate the whole plant from a single somatic cell. *In vitro* culture of cells and tissues provides a powerful way to study the developmental processes in plants.

Jelaska (1972, 1974, 1980, 1986) has established a number of pumpkin callus lines which display a rich morphogenetic potential, above all a high capacity for somatic embryogenesis. Specific culture conditions responsible for embryogenesis or organogenesis have been well defined, but the molecular basis of these phenomena remains to be elucidated. Several proteins characteristic of embryos or callus have already been identified in *Dactylis glomerata* L. (orchard grass) (Hahne *et al.*, 1988). An isoperoxidase with pI of pH 7.0 has been revealed in carrot cell suspension cultures grown under conditions stimulating embryogenesis (Joersbo *et al.*, 1989). Although cellular and biochemical markers of embryogenesis may be useful in predicting developmental events in tissue cultures, they do not explain how an embryo-specific gene expression program can be established in differentiated cells.

In plants, differentiation and growth are regulated by hormones which may be classified into five groups: auxins, cytokinins, gibberellins, abscisic acid and ethylene. Their biosynthesis, regulation and mechanism of action are still poorly understood (Meyerowitz, 1989). Plant cell transformation may be a useful tool for better understanding of genes that influence the plant hormone level and are thereby involved in developmental processes. Transformation may be achieved by *Agrobacterium tumefaciens* which induces crown gall tumors in a wide range of dicotyledonous plants, or by *A. rhizogenes*, a related pathogen responsible for hairy root disease (Hänish ten Cate, 1987; Hooykaas, 1989; Ream, 1989). Crown gall tumor cells grow *in vitro* in the absence of plant hormones due to the expression of the onc-genes present on T-DNA, a part of Ti plasmid which is integrated in plant chromosomes. *A. tumefaciens* T-DNA codes enzymes which direct auxin and cytokinin synthesis. In contrast, the root-inducing genes in T-DNA from *A. rhizogenes* do not include enzymes for auxin or cytokinin biosynthesis and may promote root development by increasing the sensitivity of transformed cells to auxins (Ream, 1989).

The research presented here concentrates largely on peroxidase as a marker enzyme in differentiation and somatic embryogenesis in pumpkin, as well as on enzyme relation to transformation and regeneration in horse-radish.

Results

Cucurbita pepo L. *(pumpkin)*

Pumpkin callus lines (established by Jelaska 1972, 1980) were tested for peroxidase activity on day 5 of an 8-day culture in the agitated liquid medium. The highest enzyme activity was deter-

Abbreviations used in this paper: AS, adenine sulfate; 2.4-D, dichlorophenoxyacetic acid; IAA, indolylacetic acid; IBA, indolylbutiric acid; NAA, naphthaleneacetic acid; MS, Murashige and Skoog; SDS, sodiumdodecyl sulfate.

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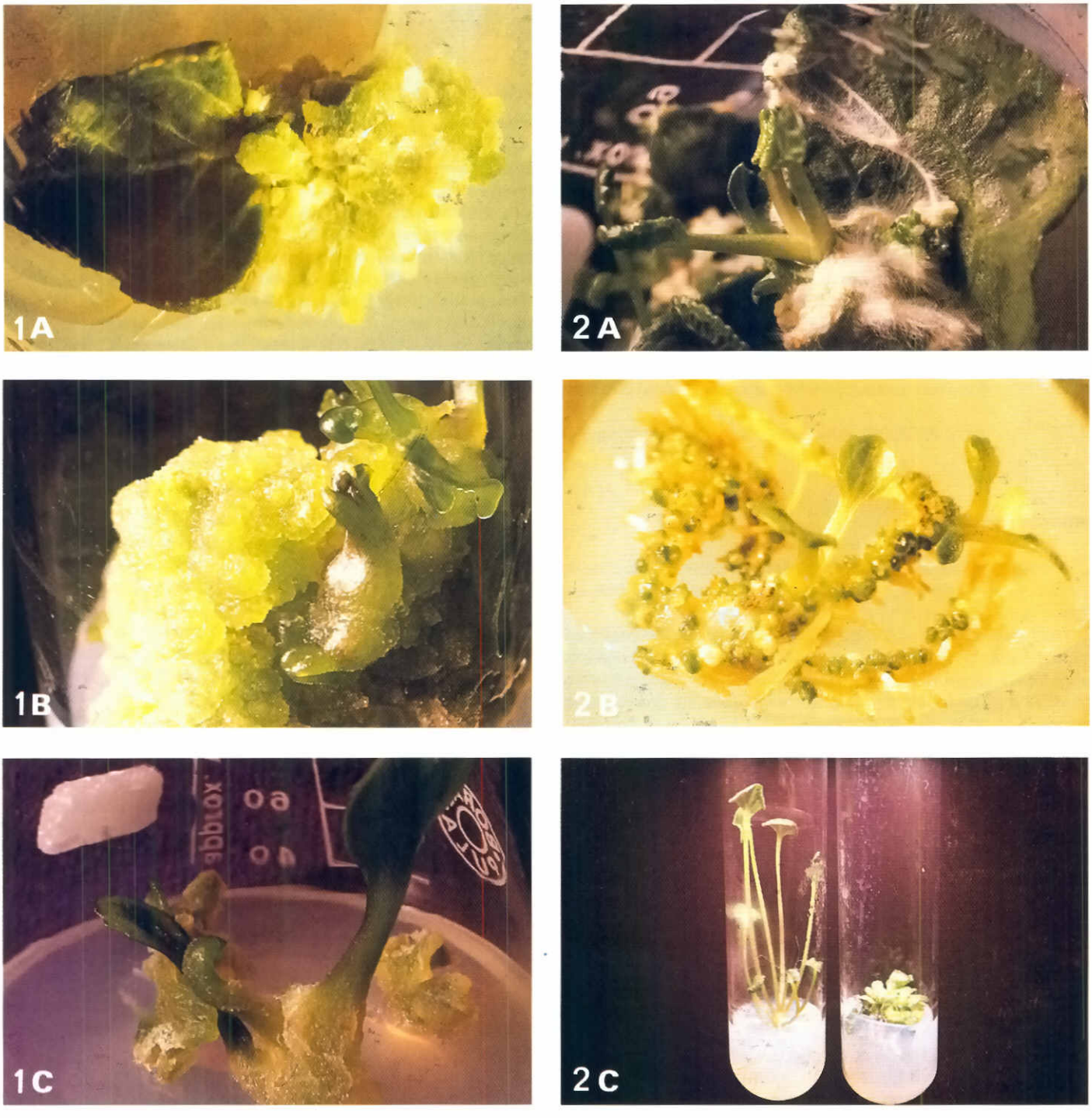

Fig. 1. *A. tumefaciens* mediated transformation of horse-radish. (A) *Primary crown gall tumor induced in leaf explant. Photographed 35 days after infection.* **(B)** *Teratogenous tumor with an embryoid consisting of two shoots on the opposite sites of its axis.* **(C)** *Tumorous tissue proliferation on the base of shoot explant derived from crown gall tissue.*

Fig. 2. *A. rhizogenes* induced hairy root transformants. (A) *Primary hairy roots induced in leaf explant. Shoots regenerated from hairy-roots. Photographed 40 days after infection.* **(B)** *Numerous adventitious buds and a few shoots thrusted out of the hairy-roots subcultured on MS medium without hormones.* **(C)** *Normal horse-radish plantlet and bushy short plant regenerated from hairy roots.*

TABLE 1

SOLUBLE PROTEIN CONTENT AND TOTAL PEROXIDASE ACTIVITY IN PUMPKIN CALLUS LINES

Callus line	Growth regulators	Protein content mg (g FW)$^{-1}$ (a)	Peroxidase activity ΔA_{470}(g FW)$^{-1}$min^{-1} (b)	Specific activity (b/a)
DE (callus)	2.4-D	0.72	2251.0	3126.3
DE (embryoids)	«	1.98	460.0	232.3
NA-3^{-1}	NAA+AS	0.62	1832.0	2954.8
Z5b/T (NE)	0	0.68	87.5	128.7
Z5b/T (E)	0	0.71	397.4	559.7

[1]Embryoids were not selected from the callus
NE, nonembryogenic culture
E, embryogenic culture
FW, fresh weight

mined in the embryogenic lines DE and NA-3 grown in the presence of growth regulators (Table 1). The minimum peroxidase activity was estimated in habituated tissue which grew without hormones and formed white aggregates with strongly reduced potential for somatic embryogenesis. An increase in peroxidase activity was registered in two of the ten culture flasks, in which the tissue became looser and after one day embryoids were noticed. Peroxidase activity was 5 to 6 times lower in selected globular embryoids than in the callus from which they originated.

Armoracia lapathifolia Gilib *(horse-radish)*

Crown gall tumors were induced in horse-radish leaf explants excised from plants propagated *in vitro*. The leaf fragments were infected with the wild octopine strain of *Agrobacterium tumefaciens* (B6S3). A large yellowish-green tumor developed in three weeks (Fig. 1A). The tumors were subcultured on MS medium without hormones. They grew mainly as unorganized tissue, or less frequently, as teratogenous tissue forming malformed embryoids and shoots (Fig. 1B). Crown gall shoots had fleshy leaves, and never rooted, only callus was developing at the base and was also bursting through the leaf surface (Fig. 1C). Infection with *A. rhizogenes* (manopine strain 8196) provoked hairy roots (Fig. 2A) which regenerated buds and shoots in a few weeks if grown in the light. Bud regeneration potency has been maintained in subsequent hairy root subcultures (Fig. 2B). The hairy root regenerants were bushy and shorter than the normal plants (Fig. 2C). They rooted on the medium with IBA as well as on the medium without hormones. To determine a tissue-specific protein pattern total soluble proteins were extracted from horse-radish normal and transformed tissues. Leaf extracts of normal and bushy transformed plants coincided in the electrophoretic protein pattern (Fig. 3). Tumor tissue derived from leaves displayed a different banding pattern with two quantitatively dominant polypeptides of low mobility (molecular weight over 85 kDa) and three in the molecular weight range of 45 to 32 kDa (Fig. 3). The polypeptide of about 19 kDa present in leaf extracts disappeared in the tumors and a new one of about 25 kDa appeared. No differences in the banding pattern were noticed between teratogenous (shoot forming) and unorganized tumors.

As shown in Table 2, total peroxidase activity per g FW in the leaf extracts of bushy transformed plants was about three times higher than in the normal leaf extracts. Enzyme activity in crown gall extracts was 6 to 7 times higher than in the normal leaves reaching its maximum in teratogenous tumors.

Discussion

Low peroxidase activity of selected embryoids and its rise prior to embryogenesis in pumpkin callus lines (Table 1) indicate that a high enzyme activity of embryogenic callus lines is hardly due to embryoids but to a tissue in which somatic embryogenesis is to be triggered. Joersbo *et al.* (1989) noticed, in carrot cell suspension culture, a rise in specific peroxidase activity a day before the globular embryoids were seen. They also revealed an isoperoxidase that correlated with the early stages of somatic embryogenesis. It seems that low peroxidase activity is a distinct characteristic of habituated tissue as it was stated for pumpkin callus lines, carrot cell lines (Joersbo, 1989) and sugarbeet calli (Hagège *et al.*, 1990).

Wild Ti plasmid induced horse-radish crown galls grew mainly in an undifferentiated way, though in about 15 percent of the cultures malformed shoots differentiated. Pumpkin crown galls induced by the same bacterial strain grew only as unorganized tumor tissue (Bakran-Petricioli and Krsnik-Rasol, 1989) while in potato only root morphogenesis was noticed (Krsnik-Rasol and Bakran-Petricioli, 1989). This illustrated that tumor morphology is not only a function of inducing plasmid but also of host plant. Crown gall may also be a mosaic of normal and transformed cells where normal cells are developmentally directed by hormones which transformed cells synthesize.

Horse-radish leaves and crown galls differed in their electrophoretic protein patterns. Differences between normal and bushy plant leaves and between unorganized and teratogenous tumors failed to be detected.

Enhanced peroxidase activity in transformed tissues might be caused by its involvement in the regulation of IAA content, which was found to be reduced in transformed potato shoots (Ooms and Lenton, 1985). High peroxidase level in tumors seems to be controversial with abundant biomass production. An inverse relationship of peroxidase activity and growth have been attributed to its ability to oxidase IAA (van Huystee and Cairns, 1980). The biosynthetic pathways of growth regulators in crown gall cells differ from the corresponding pathways of untransformed plant tissue (Hall and Thomas, 1987). Consequently, different relationships

TABLE 2

SOLUBLE PROTEIN CONTENT AND TOTAL PEROXIDASE ACTIVITY IN HORSE-RADISH NORMAL AND TRANSFORMED TISSUE

Tissue	Protein content mg (gFw)$^{-1}$ (a)	Peroxidase activity ΔA_{470}(gFw)$^{-1}$min^{-1} (b)	Specific activity (b/a)
Leaves (normal plants)	2.28	107.6	46.9
Leaves (bushy transformant)	1.56	376.0	241.0
Crown gall (unorganized)	2.22	624.0	283.6
Crown gall (teratogenous)	1.81	756.0	417.6

FW, fresh weight

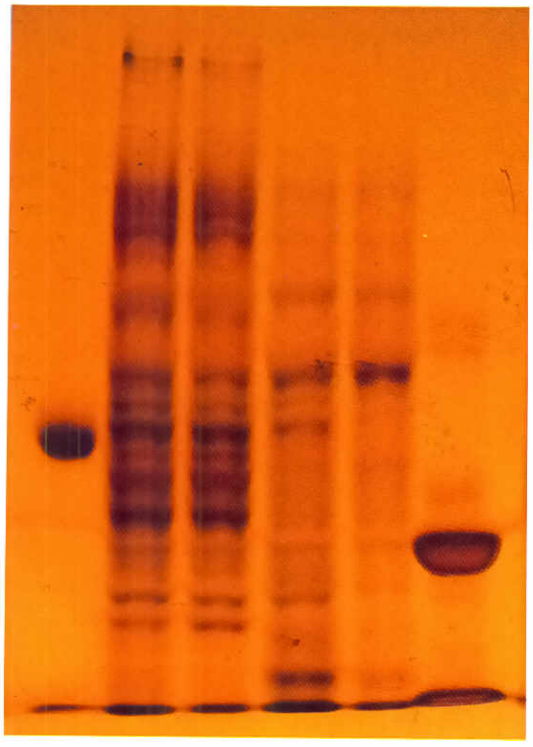

Fig. 3. Silver-stained SDS-polyacrylamide gel. *Line: 1-bovine serum albumin 68 kDa, 2-teratogenous crown gall tumor, 3-unorganised crown gall tumor, 4-leaves of bushy transgenic plant, 5-leaves of normal plant, 6-carbonic anhidrase 32 kDa.*

between peroxidase activity and growth might also be expected.

Due to its high peroxidase content horse-radish transformants should also be considered as an alternative source in peroxidase production. Yamada *et al.* (1987) recommended selected untransformed horse-radish cell lines in commercial production of peroxidase.

Leaf extracts of bushy hairy root regenerants showed higher peroxidase activity than the normal leaf extracts. A similar relationship between plant height and peroxidase activity has been reported for untransformed plants where dwarf plants had high peroxidase level (van Huystee and Cairns, 1980, Gaspar *et al.*, 1982).

Materials and Methods

Pumpkin callus lines

The pumpkin (*Cucurbita pepo* L.) callus lines were initiated and cultured on MS basal medium (Murashige and Skoog, 1962) supplemented with growth regulators as previously described (Jelaska, 1974, 1980).

The leaves of *in vitro* multiplied horse-radish plants *(Armoracia lapathifolia Gilib)* were infected with wild octopine strain of *Agrobacterium tumefaciens* (B6S3) or with manopine strain of *A. rhizogenes* (8196) according to the method of Horsch *et al.* (1985) and de Block (1988).

Transformants cultivation

All transformed tissues were cultivated on MS medium without growth regulators. Adventitious buds were induced in hairy root explants when placed in the light.

Peroxidase

Crude peroxidase extracts were prepared by grinding a fresh tissue in 0.1 M phosphate buffer (pH 7). The ml of buffer was used for 1 g of the fresh tissue. The homogenate was centrifuged at 30,000 g for 50 min. The total guayacol peroxidase activity of supernatant was determined photometrically at 470 nm. The test solution was prepared according to Siegel and Galston (1967). Enzyme activity is expressed as A_{470} (g. fresh weight)$^{-1}$ min^{-1}, or as specific activity in relation to protein content which was determined according to Bradford (1976).

Electrophoresis

Proteins were separated in SDS-polyacrylamide gels according to Laemmli (1970) and stained by silver (Blum *et al.*, 1987).

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