TRANSFORMATION OF FLAX BY AGROBACTERIUM

BY

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Dedicated to my friend, Zhengyi Lin

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SUMMARY

Regeneration of shoots from cotyledons, hypocotyls, roots and callus derived from protoplasts of Linum usitatissimum L. (flax) and Linum marginale A. Cunn. and leaf explants of L. marginale was achieved in vitro on MS medium with various combinations of growth substances. The regeneration capacity of explants from the various sources, genotypes and their responses to the various media differed considerably. Hypocotyls seemed to be the best explant source for bud regeneration followed by roots in both species. Protoplasts were readily isolated, cultured and regenerated in L. marginale but with difficulty in L. usitatissimum. The most important factor influencing bud regeneration of flax in tissue culture was the genotype. For bud regeneration from hypocotyl and root explants of the flax cultivar Bombay, the best combination of growth substances added to MS medium was 0.02mg/l NAA, 1mg/l 6-BA, 20mg/l adenine and 500mg/l cefotaxime. The cefotaxime seemed to promote the regeneration of buds from root explants. For bud regeneration in L. marginale, the optimal combination of growth substances added to MS medium was 0.02 mg/l NAA, 1mg/l 6-BA and 20mg/l adenine for hypocotyl explants, 1 mg/l zeatin for root explants and 5 mg/l zeatin for callus derived from protoplasts. These regeneration results are assessed with respect to the possible use of Agrobacterium-mediated gene transfer and other gene transfer techniques to generate genetically transformed plants of flax and L. marginale.

Based on these results, regeneration following transformation by either Agrobacterium tumefaciens carrying a disarmed Ti - plasmid vector, or Agrobacterium rhizogenes carrying an unmodified Ri plasmid, was examined in flax. Hypocotyl and cotyledon explants inoculated with A.tumefaciens formed transformed callus, but did not regenerate transformed shoots either directely or via callus. However, cotyledon explants inoculated with A. rhizogenes formed transformed roots which did regenerate

transformed shoots. Ri T - DNA encoded opines were detected in the transformed plantlets and Southern hybridization analysis confirmed the presence of T -DNA from the Ri plasmid in their DNA. Transformed plantlets had curled leaves, short internodes and some had a more developed root system characterized by plagiotropic behaviour. These results show that transformation by A. rhizogenes is an effective alternative to transformation by disarmed strains of A. tumefaciens for the genetic engineering of plants, especially when it is not possible to regenerate shoots readily from callus but is possible from roots.

A strong promoting effect on root formation by mixed inoculation of flax with A. tumefaciens strain GV3850 and A. rhizogenes strain 1855 was found. To find out the molecular nature of this effect, different strains of Agrobacterium with different combinations of chromosome background, Vir region and tzs gene were used with strain 1855 in transformation experiments. The results showed that the promoting effect was due to the tzs gene and was dependent on vir induction. The practical consequence of this result for plant genetic engineering is discussed.

In order to develop a new vector system preserving the advantages of vectors based on both the Ti plasmids and Ri plasmids but without their disadvantages, a set of new vectors has been constructed. The gene iaaH codes for indoleacetamide hydrolase which catalyzes the reaction indoleacetamide to indoleacetic acid; iaaH was subcloned from plasmid p132-10 which contains BamH1 fragment 8 of pTiA6 cloned in pBR322. The gene was either introduced into the binary vector pDG12Sa or placed under the control of the 35S CaMV promoter before being inserted in pDG12Sa. The new constructs were tested for the transformation of flax cotyledon explants on a medium containing acetamide. Transformed callus was successfully induced from inoculated explants by the new vectors and the induction of transformed callus was substrate regulated. No transformed root arising from explants inoculated with the new vectors was detected. The possible use of these constructs and their further improvement are discussed.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university and to the best of my knowledge contains no material previously published or written by another person, except where due reference is made in the text. The author consents to the thesis being made available for photocopying and loan.

Xiangcan Zhan

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ABBREVIATIONS

Ap	Ampicillin
6 - BA	6 - Benzylaminopurine
bp	Base pairs
CaMV	Cauliflower mosaic virus
Cb	Carbenicillin
Cm	Chloramphenicol
cfu	Colony forming units
2, 4 - D	(2, 4 - Dichlorophenoxy) aetic acid
DNA	Deoxyribonucleic acid
EDTA	(Ethylenedinitrilo)tetraacetic acid
GA	Gibberellic acid (Gibberellin A3)
h	Hour
IAA	Indole - 3 - acetic acid
IBA	Indole - 3 - butyric acid
kb	kilobase
Km	Kanamycin
M	Molar
MS	Murashige and Skoog medium 1962
MW	Molecular weight
NAA	1 - Naphthaleneacetic acid
nos	Nopaline synthase gene
npt - ll	Neomycin phosphotransferase ll
onc	Oncogenicity
rif	Rifampicin
Ri - plasmid	Root - inducing plasmid
Sm	Streptomycin

T - DNA	Transferred DNA
Ti - plasmid	Tumour - inducing plasmid
TL - DNA	Left transferred DNA
TR - DNA	Right transferred DNA
vir	Virulence

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CHAPTER 1 INTRODUCTION

Gene transfer through plant transformation and regeneration is revolutionizing the plant sciences because it can provide unprecedented insight into plant genetic and physiological processes; it also has great potential for use in crop improvement. Successful gene transfer in plants is mainly based on progress in two fields: 1) The development of plant cell, tissue and protoplast culture. Plants can be regenerated directly from plant tissue explants or from callus derived from plant cells or from protoplasts in vitro. 2) The development of efficient DNA transfer systems (reviewed by Cocking and Davey, 1987; by Goodman et al., 1987; and by Weising et al., 1988) including the vector systems based on Ti and Ri-plasmids of Agrobacterium (reviewed by Schell, 1987; by Bevan and Goldsbrough, 1987; by Klee et al., 1987a; and by Tepfer and Casse - Delbart, 1987), microinjection (Morikawa and Yamada, 1985; Crossway et al., 1986), direct DNA uptake (Krens et al.,1982; Paszkowsiki et al., 1984), electroporation (Shillito et al.,1985; Fromm et al. 1986) and particle bombardment (Klein et al.,1987; 1988). Today, methods of DNA transfer based on Ti or Ri-plasmids of Agrobacterium are the most developed and reliable of those available.

The principles of plant tissue culture

The principles of plant tissue culture are based on the brilliant idea, put forward by Schwann and Schleiden last century, that each living cell of a multicellular organism is totipotent (Gautheret, 1983). This led to the rapid development of plant tissue culture and was followed by:

1. The discovery and application of plant hormones and regulators, such as IAA (White, 1941; Gautheret, 1983) and kinetin (Miller et al.,1955).

2. The discovery of the regulation of organ formation in vitro (roots and shoots) by changing the ratio of cytokinin/auxin (Skoog and Tsui,1948; Skoog and Miller, 1957).

3. The regeneration of carrot embryoids from single cells in suspension culture (Steward, 1958; Gautheret, 1983).

4. The successful induction of haploid embryoids in vitro from pollen grains of cultured anthers of Datura (Guha and Maheshwari, 1966; 1967) and from isolated microspores of Nicotiana and Datura (Nitsch, 1974; 1977).

5. The isolation and culture of protoplasts and the regeneration of plants from callus derived from protoplasts (Cocking, 1960; Takebe et al., 1971); successful protoplast fusion (Carlson et al.,1972) was also a major advance.

Regeneration of plants is the key step in tissue culture. The successful regeneration of plants depends on three factors: 1) Source and location of explants, including the organ or tissue chosen, its physiological state, the size and inoculation density of explants, explant pretreatment and cultural environment (reviewed by Murashige, 1974; by George and Sherrington , 1984 and by Brown and Thorpe, 1986). 2) Medium composition, including inorganic nutrients, organic nutrients, natural nutritive mixtures and growth hormones or regulators. (reviewed by Murashige, 1974; by George and Sherrington, 1984; and by Brown and Thorpe, 1986) and 3) Genotype, which may be the key factor affecting regeneration (reviewed by Vasil, 1988; Brown, 1988).

The development of plant tissue culture offers a great possibility for genetic manipulation. Once cells or protoplasts have been genetically manipulated, they can be cultured and regenerated into plants, making the genetic engineering of plants a reality.

Crown gall and hairy root diseases

Crown gall and hairy root diseases are neoplastic diseases caused by gram negative soil bacteria which belong to the genus Agrobacterim Conn 1942. Crown gall is characterized by unorganized tumorous growths and is caused by Agrobacterium tumefaciens (Smith and Townsend 1907) Conn 1942 whereas hairy root is characterized by massive root production and is caused by Agrobacterium

rhizogenes (Riker, Banfield, Wright, Keitt and Townsend 1930) Conn 1942. A. tumefaciens can infect most dicotyledonous plants (De Cleene and De Ley, 1976), a few monocotyledonous plants (Hooykaas-van Slogteren et al., 1984; Hernalsteens et al., 1984; De Cleene, 1985) and some gymnosperms (De Cleene and De Ley, 1976). A. rhizogenes can also infect most dicotyledonous plants (De Cleene and De Ley, 1981), but it is not clear if it can infect monocotyledonous plants or gymnosperms. The virulences of A. tumefaciens or A. rhizogenes is dependent on the presence of a large plasmid, the Ti plasmid or Ri plasmid respectively (Moore et al., 1979; Van Larebeke et al., 1974). Loss of the Ti plasmid by culturing A. tumefaciens at 37 ^{0}C may result in loss of virulence (Hamilton, 1971; Van Larebeke et al.,1974). For sucessful infection, plant tissue must be wounded to allow the bacteria access to the plant cells (Riker et al., 1946). In addition, tumour and hairy root induction is prevented at temperature above 30 ^{0}C (Braun, 1958; Moore et al., 1979; Rogler, 1981).

Attachment of bacteria to the plant cell wall is also a prerequisite for virulence (Lippincott and Lippincott, 1969) and in A. tumefaciens it is controlled by two distinct linked genes, which are located on the bacterial chromosome and called chvA, chvB respectively (Douglas et al., 1985). Other loci may also be involved in attachment.

The Ti plasmid and its functions

The Ti plasmid is conjugative (Kerr et al., 1977; Genetello et al., 1977). Its molecular weight ranges from 120 x 10^6 to 150 x 10^6 Dalton, corresponding to 180 to 225 kb (Zaenen et al., 1974; Currier and Nester, 1976).

One region of the Ti Plasmid, referred to as T- DNA (transferred DNA) was shown to be transferred to plant cells and integrated into the plant nuclear DNA (Chilton et al., 1977; 1978; 1980; Willmitzer et al., 1980; reviewed by Koukoliková - Nicola, 1987 and by Zambryski, 1988) during the infection process. An imperfect direct repeat of 25 bp is present at both ends of the T- DNA (Yadav et al., 1982; Zambryski et al., 1982; Simpson et al., 1982; Holsters et al., 1983), and is the only

part of the T-DNA necessary for T-DNA transfer (Zambryski et al., 1980; Yadav et al., 1982; Wang et al., 1984); the repeats function as sites for nuclease cleavage of the T- DNA which leads to the generation of intermediate forms of T-DNA for transfer and integraion into plant DNA (Koukolikova-Nicola et al., 1985; Stachel et al., 1986; Wang et al., 1987).

Of thirteen genes that have been found in the T-DNA of Ti plasmids (reviewed by Fraley et al., 1986 and Koukoliková - Nicola et al., 1987), three have been identified as genes involved in the biosynthesis of plant hormones and responsible for oncogenicity. The ipt gene (formerly tmr) codes for isopentenyltransferase, which catalyzes the first step in cytokinin biosynthesis (Akiyoshi et al., 1984; Barry et al., 1984; Buchmann et al., 1985). The iaaM gene (formely tms-1) codes for tryptophan monooxygenase which catalyzes the reaction tryptophan to indoleacetamide (Thomashow et al., 1986) and the iaaH gene (formely tms-2) codes for indoleacetamide hydrolase which catalyzes the reaction indoleacetamide to indoleacetic acid (Schröder et al., 1983; Thomashow et al., 1984).

A novel group of compounds called opines (Petit et al., 1978), which are amino acid-keto acid conjugates or phosphorylated sugar derivatives (Ellis and Murphy, 1981), are found only in infected plant cells and is genetically controlled by the plasmids of Agrobacterium (Bomhoff et al., 1976). They are encoded by genes located on T-DNA in the tumour cells (Montoya et al., 1977; Petit et al., 1978; Schell et al., 1979; Tempé and Goldmann, 1982). An opine can be used as a supply of nutrients by the tumour inducing strain of Agrobacterim (Petit et al., 1978; Schell et al., 1979) and the presence of opines can serve as a means of identifying plant transformation. The genes responsible for opine catabolism are located on the non-transferred portion of the Ti plasmid (Petit et al., 1978; Holsters et al., 1982).

Another region of the Ti plasmid, referred to as the virulence (Vir) region, of about 35 kb is also essential for pathogenicity. The Vir region is not transferred but is involved in the early stages of infection (Hille et al., 1984; Hooykaas et al., 1984). The expression of vir genes has been proved to be activated by some small phenolic

compounds, such as acetosyringone, a-hydroxyacetosyringone and others, exuded from cultured plant cells or wounded tissues (Stachel et al., 1985). Induction is pH and temperature dependent (Alt-Moerbe et al., 1988). Six operons were found in the Vir region of the octopine type Ti plasmid pTiA6 and in the nopaline type Ti plasmid pTiC58. They are virA, virB, virC, virD, virE and virG (Stachel and Nester, 1986; Rogowsky et al., 1987). Another operon ,virF, was found in the octopine Ti plasmid pTiAch5 but neither in pTiC58 nor in pTiA6 (Hooykaas et al.,1984).

It has been proposed that the inducing chemical interacts with the vir A product, which is a cytoplasmic membrane bound protein, and then the changed virA protein converts the virG protein to an active form. The activated virG protein will in turn incite the remaining vir loci (Winans et al., 1986; Leroux et al., 1987), in which virD codes for an endonuclease nicking within the T-DNA borders (Yanofsky et al., 1986), leading to the generation of the T-strand, a single-strand intermediate form of T-DNA for transfer to the plant (Stachel et al., 1986).

It has been proposed that two other genes, iaaP and tzs, located outside T-DNA, are also related to the virulence function in the nopaline type plasmid; iaaP is necessary for IAA production (Liu et al., 1982) and tzs gene for trans-zeatin and trans-ribosylzeatin production (Beaty et al., 1986). Further research work reveals that the tzs gene is also present in the Vir region of the agropine type and mannopine type Ri plasmids, but not in the octopine Ti plasmid or the agropine Ti plasmid pTiBo542. It is under the control of the virA and virG regulatory loci, which implies a role in tumour induction (Akiyoshi et al., 1987; Alt-Moerbe et al., 1988; John and Amasino, 1988; Powell et al., 1988).

The Ri plasmd and its function

The Ri plasmid can be considered a different type of Ti plasmid with many similarities. It is a plasmid with the same size range as the Ti plasmid (White and Nester, 1980a,b), and shares extensive homology with the Ti plasmid in the Vir region (Huffman et al., 1984; Jouanin, 1984). Like the Ti plasmid, its T-DNA is flanked by

the 25 bp border sequences and will transfer to plant cells, integrate into plant genomic DNA and encode the synthesis of opines (White et al., 1982). Three different types of Ri plasmid have so far been identified corresponding to the synthesis of different opines: agropine type, mannopine type and cucumopine type (Petit et al., 1983; Petit and Tempé, 1985; Brevet et al., 1988). On the other hand, Ryder et al. (1985) observed that strains of A. rhizogenes differed in their capacity to induce root proliferation on carrot root discs. Strains containing mannopine and cucumopine type plasmids are able to induce root growth only on the apical surfaces of discs and strains containing agropine type plasmids induce root proliferation on both apical and basal surfaces. They are referred to as polar and nonpolar strains respectively. It has been found that the agropine type Ri plasmids carry two distinct T- regions, TL and TR (Jouanin, 1984; De Paolis et al.,1985; White et al., 1985), whereas mannopine and cucumopine type Ri plasmids carry only the TL region (Koplow et al., 1984; Cardarelli et al., 1985; Filetici et al., 1987; Combard A and Baucher M, 1988). The mannopine and cucumopine synthesis genes as well as rol genes have been localized in TL-DNA, and the agropine synthesis genes as well as auxin synthesis genes in TR-DNA (Huffman et al., 1984; De Paolis et al., 1985; Cardarelli et al., 1985; and White et al., 1985). Although the functions of rol genes are not yet thorougly understood, it has been proposed that they confer a prerhizogenic state on transformed cells while the auxin produced by the auxin genes triggers differentiation of these cells into roots (Spena et al., 1987; Vilaine and Casse-Delbart, 1987a; Cardarelli et al., 1987; Schmülling et al., 1988). Further studies indicated that the rol genes of the TL-DNA including rolA, rolB, rolC, and rolD, are synergistic during hairy root initiation. The rolB product might render plant cells more sensitive to the action of auxin, whereas the rolC product would counterbalance its activity. The combined activity of rol genes makes transformed plant cells much more sensitive to auxin and they tend to form roots, (Cardarelli et al., 1987; Schmülling et al., 1988). In polar strains, the auxin is normal plant auxin which accumulates at the apical surface and causes polarity of root formation.

Vectors and transformation techniques

A variety of vectors, including cointegrate vectors and binary vectors based on Ti plasmids, have been constructed and used to introduce foreign DNA into plant cells (Pouwels et al., 1985; Klee et al., 1987a; Bevan and Goldsbrough, 1987). Transformed cells grow into callus under selective pressure for a phenotype such as kanamycin resistance conferred by a gene carried on the T-DNA and are then regenerated into transformed plants.

pGV3850 is a typical example of a cointegrate vector. It is based on Ti plasmid pTiC58 in which the entire T- DNA, except for a small border region and the nopaline synthase gene, was substituted by pBR322. Foreign DNA inserted in pBR322 can transform or be mobilized into Agrobacterium containing pGV3850 where homologous recombination between pBR322 regions of these two plasmids leads to an integration of the foreign DNA into pGV3850 between two T-DNA borders. The new cointegrate can be used to introduce the foreign DNA into plant cells (Zambryski et al., 1983).

The development of binary vectors was based on the finding that the Vir locus of the Ti plasmid acted in trans to T-DNA borders (Hoekema et al., 1983). It has an important advantage over a cointegrate vector because it avoids the recombination step which can interfere with potentially unstable passenger DNA and lower the transfer efficiency of plasmid from E.coli to Agrobacterium (Fraley et al., 1986; Bevan and Goldsbrough, 1987). Binary vectors are based on wide host range plasmids. Examples are: Inc P, pVS1, Inc Q, Inc W, or plasmid pArA4a (Ditta et al., 1980; Matzke and Matzke, 1986; Machida et al., 1986; Deblaere et al., 1987 Vilaine and Casse-Delbart, 1987b), which can be easily mobilized from E.coli to Agrobacterium and can be maintained, and replicated in both E.coli and Agrobacterium. They also contain both left and right borders and are constructed with several unique restriction sites, an antibiotic gene and a marker gene for identification and selection. Foreign DNA inserted into a binary vector is usually transformed into E. coli and then transferred by triparental mating into Agrobacterium containing a compatible plasmid

with the trans-acting Vir function, and then transferred into plant cells (Fraley et al., 1986; Bevan and Goldsbrough, 1987). The first plant transformation and regeneration was achieved using Ri plasmid (Chilton et al., 1982) and recently, interest in vectors derived from the Ri plasmid has increased (Tepfer and Casse-Delbart, 1987). In several different species, transformed plants have been regenerated from hairy roots induced by A. rhizogenes (Tepfer and Casse-Delbart, 1987; Zhan et al., 1988).

Two major techniques are available to obtain transgenic plants by Agrobacterium. They are the co-cultivation method and the leaf-disc method. The co-cultivation method was first developed by Marton et al.(1979). The procedure is: cells just derived from protoplasts are inoculated with Agrobaterum and co-cultured for a short period. Transformed callus will grow up under selection pressure and produce transgenic plants. Since only a few species have the ability to regenerate plants from callus derived from protoplasts, this method is very limited. On the other hand, the leaf-disc method is widely used because of its simplicity and efficiency. It was developed by Horsch et al. (1985). In the procedure, axenic or surface-sterilized leaf, cotyledonous pieces or explants from other sources are inoculated and co-cultured with A. tumefaciens or A. rhizogenes for about two days. Then they are transferred to medium containing antibiotics, for callus growth, root initiation or shoot regeneration. Regenerated shoots should be further analysed to confirm transformation because escape of non-transformed shoots from selection pressure frequently occurs. Generally, identification of products by marker genes, resistant characters conferred by genes introduced in the regenerated plants or, the most reliable method, DNA hybridization can be used to identify transgenic plants (Horsch et al., 1985). Experiments show that foreign DNA inserted into genomic DNA of transformed plants is stable and shows Mendelian inheritance following sexual reproduction (Wallroth et al., 1986).

Flax

Flax (Linum usitatissimum L.) is agronomically important as a source of natural fibres and industrial oil, and may become important as a source of edible oil and protein (Green and Marshall, 1984). It is also scientifically important because of the well characterized genetic control of host-parasite interaction between flax and its rust (Melampsora lini [Ehrenb.] Lév), elucidated by the pioneering work of Flor (1956). Genetic manipulation of flax may be useful both in improving the agronomic qualities of flax and in elucidating the molecular basis of the genetic interaction between flax and its rust. Flax is a dicotyledonous plant amenable to transformation by A. tumefaciens or A. rhizogenes. Flax easily regenerates from hypocotyl and stem explants (Gamborg and Shyluk, 1976; Link and Eggers, 1946; Mathews and Narayanaswamy,1976; Murray et al., 1977) and with less ease from cotyledon explants (Mathews and Narayanaswamy, 1976; Rybczynski, 1975). So it should be possible to regenerate flax plants that have been transformed by Agrobacterium. Recently, Basiran et al. (1987), Jordan and McHughen (1988) reported the regeneration of transformed flax plants from transformed callus or hypocotyl explants inoculated with a disarmed strain of A. tumefaciens. Unfortunately, no data showing npt-II activity or the presence of T-DNA in transformed shoots are presented.

Linum marginale

Wild relatives of cultivated plant species are often useful sources of agronomically valuable genes, or serve as useful tools in the genetic manipulation of crop species for research purposes. Flax has more than 150 wild relatives in the genus Linum (Winkler 1931; Wang 1935). Some of these have already served as useful sources of agronomically valuable genes, e.g. genes for resistance to flax rust (Melampsora lini (Ehrenb.) Lév.) (Wicks and Hammond 1978). Linum marginale A. Cunn. is a wild relative of cultivated flax that is native to Australia. This native flax has potential agronomic qualities, since Australian Aborigines used its stems as a source of fibre and its seeds as a source of food (Gott 1985). It may also serve as a

source of fibre and its seeds as a source of food (Gott 1985). It may also serve as a useful source of genes for resistance to flax rust, since rust isolates from L. marginale are generally avirulent on L. usitatissimum, and vice versa (Kerr 1959), presumably indicating a different and possibly useful spectrum of resistance genes in native flax. No work has yet been done on tissue culture and plant regeneration of L. marginale.

Scope of this study

This thesis is divided into 7 Chapters. Chapter 1 and Chapter 7 are the introduction and general discussion parts respectively.

Chapter 2 and Chapter 3 deal with the potential of shoot regeneration from different sources of explants of Linum usitatissimum and Linum marginale with a view to their transformation with Agrobacterium or other methods of gene transfer.

Chapter 4 examines the possibility of transforming flax with Agrobacterium. Using the leaf-disc method, explants from different sources of flax were inoculated with A. tumefaciens or A. rhizogenes. Regeneration of flax following transformation by A. rhizogenes was achieved.

Chapter 5 describes the strong effect of tzs gene from Ti plasmid pTiC58 on promoting root induction by agropine strain 1855 of A. rhizogenes. As a result, a new method of transformation has been proposed.

Chapter 6 focuses on the construction of a new binary vector system, by introducing the iaaH gene from pTiA6 into the binary vector pDG12Sa, which is substrate regulated and induced transformed callus from flax cotyledon explants. The possible use of these constructs and their further improvement are discussed.

The project was part of a wider study aimed at cloning a flax gene encoding resistance to flax rust (Jones et al., 1985).

CHAPTer 2 IN VITRO PLANTLET FORMATION IN FLAX

Introduction

In vitro plantlet formation in flax has been previously achieved. Mathews and Narayanaswamy (1976) reported the regeneration of buds on hypocotyl segments; the development of buds was promoted by 1ppm NAA or 1ppm IBA added to MS (Murashige and Skoog, 1962) medium. The regeneration of buds from hypocotyl callus was achieved on MS medium containing 1ppm IAA or 2-4ppm IBA and 1ppm kinitin. They found that 20ppm adenine used in conjunction with kinetin stimulated bud initiation. Gamborg and Shyluk (1976) also reported the induction of buds from hypocotyl segments and hypocotyl callus but on media with different hormone combinations. They found that MS medium with 0.1 μM IAA or NAA and/or 1-10 μM zeatin enhanced bud initiation and shoot development on the hypocotyl explants; MS medium with 1-10 μM 6-BA promoted bud regeneration from hypocotyl callus. It was found that buds developed from hypocotyls were of epidermal origin (Link and Eggers, 1946). Bud regeneration from cotyledon explants was also achieved on MS medium containing 1ppm NAA and 10 ppm kinetin but with much less ease (Ryczyñski , 1975). Barakat and Cocking (1983) successfully regenerated plantlets from callus derived from protoplasts of flax. They isolated protoplasts from root, hypocotyl and cotyledon of flax seedlings and regenerated buds from protoplast-derived callus of root and cotyledon origin separately on MS medium with 0.1 mg/l NAA and 0.5 mg/l 6-BA or MS with 0.02 mg/l NAA and 1.13 mg/l 6-BA.

In this chapter, the shoot regeneration properties of flax from different sources of explants and varieties were examined with a view to the transformation of flax by Agrobacterium or other methods of gene transfer.

Materials and methods

Plant Material

Ten cultivars of flax, Abyssinian, Akmolinsk, B^{14} x Barke, Bison, Bombay, Hoshangabad, L2, Precederia, Polk and Stewart were used. These cultivars were chosen because they have diverse geographic or varietal backgrounds, and so, presumably diverse genetic backgrounds, a factor of importance in shoot regeneration. Seeds were surface sterilized for 30 min with 3% sodium hypochlorite containing 0.01% Tween 80, washed several times with sterile distilled water, then germinated and grown on hormone free MS (Murashige and Skoog, 1962) medium at 25 ^{0}C under a 16 h light 500 - 1000 lux/8 h dark cycle.

Shoot regeneration from hypocotyl explants

Hypocotyl segments, 0.8 - 1.0 cm in length, from 7 day old seedlings of all ten cultivars were used. Explants were placed on MS medium supplemented with various growth regulators (Table 2-1) and cultured at 25 ^{0}C under a 16 hour light 1000 lux/8hour dark cycle. Each treatment consisted of 25 replicates and repeated at least twice. Results were recorded after 3 weeks of culture.

Shoot regeneration from cotyledon explants

Cotyledon explants, 0.5 x 0.5 cm in size, from 7 day old seedlings of Akmolinsk, Bombay, L2 and Precederia were used. Explants were placed on MS medium supplemented with various growth regulators (Table 2-2) and cultured as above. Each treatment consisted of 10 replicates and repeated at least twice. Results were recorded after 45 days.

Shoot regeneration from root explants

Five cultivars of flax, Akmolinsk, Bombay, B^{14} x Barke, Hoshangabad and Polk, were used. Cotyledons, from 7-10 day old seedlings, were excised, bisected transversely and cultured on MS medium containing 0.5 mg/l NAA to induce roots. The roots that developed on the cotyledons were excised when 0.5-1 cm in length, placed on various differentiation media (Table 2-3) and incubated as above. Twenty explants were used for each experiment and all experiments were repeated at least three times.

Protoplast isolation, culture and shoot regeneration

Protoplasts were isolated from cotyledons of 7 day old seedlings of Bombay and Precederia. Cotyledons were cut into approximately 1mm wide strips and plasmolysed in plasmolysis solution (0.375 M mannitol,.0.05 M calcium chloride) for 1 hour, submerged in plasmolysis solution containing 2% cellulase (Onozuka R-10, Yakult Phamaceutical Industry Co. Ltd., Nishinomiya, Japan), 0.5% macerozyme (R-10, Yakult Honsha Co. Ltd., Tokyo, Japan) and 0.1% Pectolyase (Y-23, Seishin Pharmacetical Co., Ltd., Koamicho, Nihonbashi, Tokyo, Japan), vacuum infiltrated at -85 kPa for 3 - 5 min, and incubated at 25 ^{0}C for 16 hours. After incubation, the tissue was gently squeezed and 3 vol. 0.21 M calcium chloride was added to the mixture which was then filtered through a 70 µm mesh stainless steel sieve. Protoplasts were pelleted by centrifugation at 100 x g for 5 min, washed once with 0.21 M calcium chloride and pelleted again. They were then resuspended in MSP-1 or MB5P (Barakat and Cocking, 1983) liquid medium at a density of 5 x 10^4 ml/l and cultured in 5 cm plastic Petri dishes, as 1 ml protoplast suspensions over 4 ml MSP-1 or MB5P solidified with 0.5 % agarose, at 25 ^{0}C under a 16 h diffuse light / 8 h dark cycle. After one month of incubation, the solidified agarose media with microcalli growing on them were each cut into four pieces and transferred to 9 cm plastic Petri

Table 2-1. Supplements added to MS medium in various differentiation media and their effect on bud initiation on hypocotyl explants of the flax cultivar Akmolinsk.

Medium	Supplements (mg/l)				Bud number per explant*
	IAA	NAA	6-BA	Adenine	
MS	0	0	0	0	5.8±0.5
I 0.02	0.02	0	0	0	3.6±0.5
I 0.5	0.5	0	0	0	1.26±0.4
I1	1	0	0	0	0.7±0.2
N 0.02	o	0.02	0	0	5.5±0.5
B 0.5	0	0	0.05	0	14.5±1.2
B 1	0	0	1	0	19.0±1.6
IB	0.02	0	1	0	12.8±1.3
IBA	0.02	0	1	20	18.6±1.3
NB	0	0.02	1	0	15.4±1.2
NBA 20	0	0.02	1	20	22.3±1.9
NBA 50	0	0.02	1	50	19.9±2.3
NBA 100	0	0.02	1	100	13.1±1.3

* Bud number per explant ± S.E.M.

Table 2-2. Supplements added to MS medium in various differentiation media and their effect on bud initiation on cotyledon explants of four flax cultivars, Akmolinsk, Bombay, L_2, Precederia.

Medium	Supplements (mg/l)					Bud initiation*			
	IAA	NAA	6-BA	Zeatin	Adenine	Akmolinsk	Bombay	L_2	Precederia
B1	0	0	1	0	0	5.0±3.5	25.0±3.5	0	35.0±3.5
B2	0	0	2	0	0	32.5±7.8	40.0±7.1	0	5.0±3.5
B5	0	0	5	0	0	16.7±8.7	15.0±3.5	0	0
Z1	0	0	0	1	0	0	5.0±3.5	0	-
Z2	0	0	0	2	0	35.0±3.5	0	0	-
Z5	0	0	0	5	0	35.0±3.5	0	0	-
NB	0	0.02	1	0	0	5.0±3.5	30.0±7.1	0	10.0±5.0
IB2	0.5	0	2	0	0	13.3±3.3	55.0±3.5	0	-
IB5	0.5	0	5	0	0	10.0±7.1	40.0±7.1	10.0±5.0	-
NBA	0	0.02	1	0	20	27.1±11.2	25.0±3.5	-	0
NB5A	0	0.02	5	0	20	16.7±6.5	-	-	-

* Percentage of explants with buds ± S.E.M.

- Not tested.

Table 2-3. Supplements added to MS medium in various differentiation media and their effect on growth of callus and bud initiation on root explants of the flax cultivar Bombay.

Medium	Supplements (mg/l)				Effect on	
	NAA	6-BA	Adenine	Cefotaxime	Callus growth[1]	Bud initiation[2]
MS	0	0	0	0	-	0
B	0	1	0	0	++	9.0±1.5
NB	0.02	1	0	0	+++	5.0±2.4
NBA	0.02	1	20	0	+++	11.9±3.8
NBACf	0.02	1	20	500	++	22.0±3.2
N0.5BA	0.5	1	20	0	++++	1.7±1.4
B2	0	2	0	0	+	1.3+1.2
NB2	0.02	2	0	0	+	3.3±2.9
NB2A	0.02	2	20	0	++	6.7±1.4
B5	0	5	0	0	+	0
NB5	0.02	5	0	0	+	0
NB5A	0.02	5	20	0	+	0
B10	0	10	0	0	-	0
NB10	0.02	10	0	0	-	0

1 - = no callus, + = poor callus growth on some explants, ++ = poor callus growth on all explants, +++ = good callus growth on all explants ++++ = very good callus growth on all explants. 2 Percentage of explants with buds ± S.E.M.

Table 2-4. Supplements added to MS medium in various differentiation media and their effect on bud initiation on callus developed from protoplasts of the flax cultivar Precederia.

	Supplements (mg/l)					Bud initiation	
Medium	IAA	NAA	6-BA	Zeatin	Adenine	callus number cultured	callus number initiated buds
B0.5	0	0	0.5	0	0	40	2
B1	0	0	1	0	0	38	0
NB0.5	0	0.02	0.5	0	0	20	0
NB	0	0.02	1	0	0	116	2
NBA	0	0.02	1	0	20	96	0
NZ	0	0.02	0	1	0	20	0
NZA	0	0.02	0	1	20	20	0
I0.5B0.5	0.5	0	1	0	0	26	0
I0.5B2	0.5	0	2	0	0	60	0
I0.5B5	0.5	0	5	0	0	40	0
I0.5BA	0.5	0	1	0	20	40	0

dishes each containing 6 ml NB medium (Table 2-4), two pieces per plate. After another 20-30 days of incubation, microcalli 0.5 - 0.8 cm in diameter were transferred to various differentiation media for shoot regeneration (Table 2-4).

Results

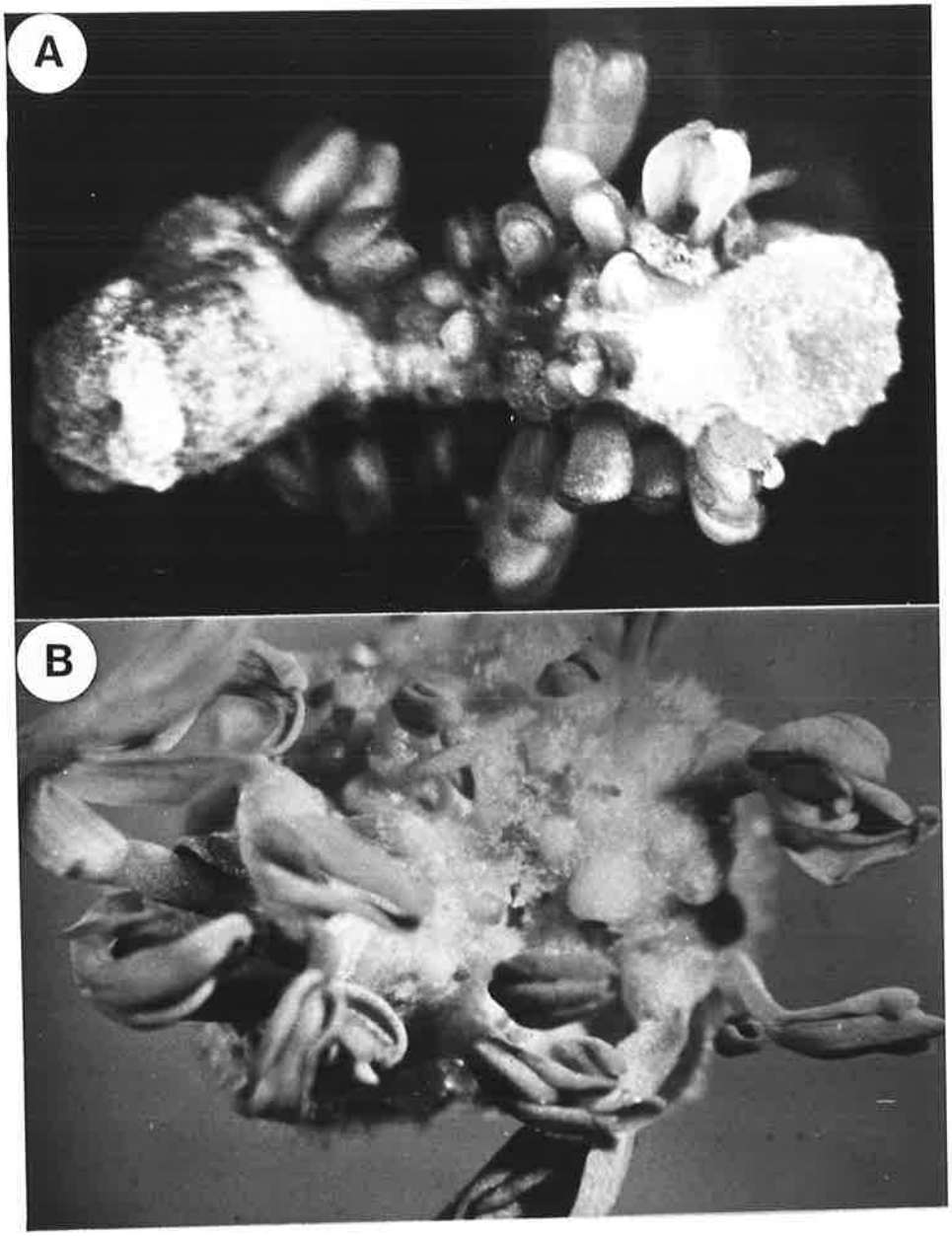
Hypocotyls

In the case of Akmolinsk, bud primordia were first visible by microscopy after 3 days culture and buds were observed along the length of hypocotyl explants after 7 days but with higher frequency near the cut ends (Fig. 2-1A). Many buds arose directly from the surface of the explants without forming any callus, while some arose on nodules which developed from the explants. Bud regeneration occurred on all media (Table 2-1), even in the absence of added phytohormones. The addition of cytokinin or cytokinin plus adenine in conjuction with auxin stimulated bud formation, not only by increasing the frequency of sites of bud initiation, but also by promoting the formation of clusters of buds at these sites. However, high concentration of adenine (50-100 mg/l) inhibited bud initiation. After 10-15 days of culture, small amounts of callus had developed at the cut ends of the explants, but few buds arose from this callus. Hypocotyl explants from nine other cultivars were also tested on NBA medium. They all showed very good and almost equal regeneration ability.

Cotyledons

After 10-15 days of culture, cotyledon explants had swollen a little, developed callus from the cut ends and numerous nodules on their surface. Buds appeared after 25 days of culture on various media (Table 2-2). Most buds arose from nodules and a few from the cut ends (Fig. 2-1B). Both 6-BA and zeatin stimulated bud initiation, but 6-BA seemed better than zeatin in promoting callus growth. Auxin and adenine used in additioin to 6-BA did not obviously stimulate bud initiation. Among the four cultivars of flax that were tested, Akmolinsk and Bombay showed good regeneration abilities and L2 was the worst .

Fig. 2-1. Bud regeneration from explants of various sources of flax. A. From hypocotyl. B. From cotyledon. C. From root.



C

Roots

Among the five cultivars of flax that had been tested, Bombay showed the best regenertation of buds from root explants. Of the others, only Akmolinsk showed bud regeneration on one medium (NB), and the remainder showed no regeneration on any of the media (data not shown).

Root explants of the flax cv.Bombay started swelling after 10 days of incubation and buds began appearing after 18 days (Fig. 2-1C). The effects of the various differentiation media on callus growth and bud formation on root explants are shown in Table 2-3. 6-BA at 1 mg/l stimulated callus growth and bud initiation, but depressed callus growth and bud formation at high concentration. NAA at 0.02 mg/l had no significant influence on callus growth and bud initiation, but stimulated callus growth and bud initiation at 0.5 mg/l. Adenine in the presence of 6-BA stimulated callus growth and bud initiation, whereas the additional presence of cefotaxime repressed callus growth, but further increased bud regeneration. The difference between bud initiation on root explants cultured on NBA and NBACf media was significant ($P < 0.05$), but buds occurred 7days later on NBACf than on NBA.

The combination of 0.02 mg/l NAA, 1 mg/l 6-BA and 20 mg/l adenine (NBA or NBACf) was best for bud initiation, and 0.5 mg/l NAA, 1 mg/l 6-BA and 20 mg/l adenine (N0.5 BA), best for callus growth.

Protoplasts

Protoplasts were not readily isolated from cotyledons, but looked healthy. First divisions of protoplasts were observed within 48 hours. In Precederia the resulting calli grew to 0.1 - 0.2 cm in diameter within 1 month and the calli continued growing after transfer to reach 0.5 - 0.8 cm in another month. Upon tranfer to regeneration media, 6-BA alone was found to be sufficient to maintain callus growth, but NAA in addition to 6-BA greatly enhanced callus growth (Table 2-4). Bud regeneration

occurred only on two media (B 0.5 and NB) with very low frequency. In Bombay, no callus grew up big enough for transfer and regeneration.

Discussion

In vitro shoot regeneration of flax was satisfactorily achieved from various explants and varieties. Two types of bud regeneration were observed, either direct from the surface of explants or via callus.

Direct bud regeneration occurred very rapidly and efficiently on the surface of hypocotyl explants in which buds arise directly from epidermal cells (Link and Eggers, 1946). This kind of regeneration would be a good means of clonal multiplication. It is a rare phenomenon , occurs in at least two species of the genus Linum (Chapter 3), is not thoroughly understand and would be worth further investigation.

Mathews and Narayanaswamy (1976) tested the effect of adenine on bud initiation callus on flax hypocotyls, and found that by itself it was less effective than kinetin, but in conjunction with kinetin stimulated bud initiation. Our experiments confirm this effect of adenine in conjunction with another cytokinin, 6-BA on bud initiation from root or hypocotyl explants. There are at least two possible explanations for this phenomenon. 1) Adenine may affect plant cells by enhancing the effect of exogenous cytokinin. 2) Adenine, which is a biosynthetic precursor of cytokinins (Koshimizu and Iwamura, 1986), may enhance the de novo biosynthesis of endogenous cytokinins, and so supplement the effect of exogenous cytokinin. Endogenous cytokinins affect plant cells differently from exogenously supplied cytokinin (Binns et al., 1987), which may explain why the same effect cannot be achieved simply by adding more exogenous cytokinin (see Table 2-1).

Mathias and Boyd (1986) reported the stimulating effects of cefotaxime in embryogenesis and regeneration in wheat. Cefotaxime in the presence of 6-BA and adenine also increased bud initiation from root explants of flax (Table 2-1). Therefore, the stimulating effect of cefotaxime on regeneration is not limited to monocotyledonous plants, but also occurs in at least one dicotyledonous plant. On

the other hand, cefotaxime stimulated callus growth of wheat but depressed callus growth and delayed bud initiation in flax. So, the effects of cefotaxime on callus of the two species differ. The mechanism of cefotaxime action is not known. However, it is fortuitous that cefotaxime enhances bud regeneration since it is commonly used at the same concentration to suppress agrobacterial growth in plant transformation experiments, without regard to its effects on regeneration.

It appears that one of the most important factors influencing regeneration of flax in tissue culture is plant genotype. For example, root explants of only one out of five cultivars regenerated buds consistently and it did so on a wide range of media. Therefore, to obtain satisfactory regeneration, the choice of an appropriate genotype of flax appears to be even more important than the choice of culture conditions. The influence of genotype on the ability of plants to regenerate in vitro has been pointed out previously (Hughes, 1981; Tomes and Smith, 1985) and Lu et al. (1983) and Duncan et al. (1985) have shown that the ill effects of some genotypes can be compensated to some extent by varying the culture conditions of either the source material or the explants.

The hypocotyls seemed to provide an ideal system for transformation experiments with Agrobacterium containing a Ti - plasmid. Because: 1) bud regeneration occurred very rapidly and efficiently, 2) most buds developed from epidermal cells which might provide easy access to infection by Agrobacterium. However, it may be useful for genetic transformation of flax using DNA particle gun technique (Klein et al., 1987). Since regeneration of buds on callus developed from cut ends of cotyledon explants is quite rare, cotyledon explants seemed unsuitable for transformation experiments with Agrobacterim because Agrobacterium only infects cells at wound sites. Also, the regeneration frequency of calli derived from protoplasts was too low for routine transformation with Agrobacterium or with other methods of gene transfer. However, roots seemed to have considerable potential because: 1) the origin of regenerating buds from callus would not matter, if the roots themselves had been induced by A. rhizogenes, since the entire root would be

genetically transformed, 2) the regeneration of buds from root explants was very efficient.

CHAPTER 3 IN VITRO PLANTLET FORMATION IN LINUM MARGINALE

Introduction

In vitro plantlet formation in L. marginale has not been reported before. Barakat and Cocking (1985) did some work on the isolation and culture of protoplasts of several wild Linum species. They found that the regeneration ability of plants of different species varied considerably. Out of six species, plantlets of two species were successfully regenerated from callus derived from protoplasts.

In this Chapter, the regeneration properties of L. marginale from different sources of explants were examined with a view to their transformation by different methods of gene transfer.

Materials and methods

Plant Material

Seeds of Linum marginale isolate LMKAP18 were kindly provided by G. J. Lawrence who collected them from a single wild plant growing in Mt Kaputar National Park, N.S.W. The seeds were surface disinfected, then soaked for 3 days in 300 ppm aqueous GA3 at 4 ^{0}C to break their dormancy (G. J. Lawrence, unpublished). The seeds were then germinated and the seedlings grown on MS (Murashige and Skoog 1962) medium under a 16 h light 500 -1000 lux / 8 h dark cycle at 25 ^{0}C. Continuous shoot cultures were initiated by excising shoot tips 0.5 -1 cm in length and growing them as above. The cultures were subcultured once a month.

Tissue Culture and Shoot Regeneration

Cotyledon explants 0.3 x 0.3 cm, hypocotyl segments 0.5 - 1 cm long, and root segments 0.5 - 1 cm long from 5 day old seedlings and leaf explants from shoot cultures were cultured as above on various media (Table 3-1) for bud regeneration.

Protoplast Isolation, Culture and Shoot Regeneration

Protoplasts were isolated from shoots taken from shoot culture, some of which were kept in the dark for 1 - 2 days prior to protoplast isolation in an attempt to improve protoplast yield. Shoots 4 - 5 cm in length were cut longitudinally, plasmolysed in plasmolysis solution (0.375 M mannitol,.0.05 M calcium chloride) for 1 h, submerged in plasmolysis solution containing 1% cellulase (Onozuka R-10, Yakult Pharmaceutical Industry Co. Ltd., Nishinomiya, Japan) and 0.1% macerozyme (R-10, Yakult Honsha Co. Ltd., Tokyo, Japan), vacuum infiltrated at -85 kPa for 3 - 5 min, and incubated at 25 ^{0}C for 16 h. After incubation, the tissue was gently squeezed and 3 vol. 0.21 M calcium chloride was added to the mixture which was then filtered through a 70 μm mesh stainless steel sieve. Protoplasts were pelleted by centrifugation at 100 x g for 5 min, washed once with 0.21 M calcium chloride and pelleted again. They were then resuspended in MSP-1 (Barakat and Cocking, 1983) liquid medium at a density of 2 x 10^5 /ml and cultured in 5 cm plastic Petri dishes, as 1 ml protoplast suspensions over 4 ml MSP-1 solidified with 0.5 % agarose, at 25 ^{0}C under a 16 h diffuse light / 8 h dark cycle. After 1 month of incubation, the solidified agarose media with microcalli growing on them were each cut into four pieces and transferred to 9 cm plastic Petri dishes each containing 6 ml NB medium (Table 3-2), two pieces per plate. After another month of incubation, microcalli 0.5 - 0.8 cm in diameter were transferred to various differentiation media for shoot regeneration (Table 3-2).

Regeneration of Rooted Plantlets

Shoots longer than 1 cm that arose from the various sources above were excised and transferred to MS medium for rooting.

Table 3-1. The effect of various culture media on shoot regeneration on explants from various sources of L. marginale.

Medium[a]	Cotyledon explants producing shoots		Hypocotyl explants producing shoots		Root explants producing shoots		Leaf explants producing shoots	
	Out of 20	%	Out of 20	%	Out of 20	%	Out of 20	%
MS	0	0	20	100	0	0	nt	–
B1	8	40	20	100	0	0	nt	–
B2	8	40	17	85	0	0	nt	–
B5	died	–	died	–	died	–	nt	–
NB	9	45	20	100	0	0	nt	–
NBA	14	70	20	100	0	0	nt	–
Z1	0	0	20	100	20	100	6	30
Z2	0	0	20	100	17	85	nt	–
Z5	6	30	11	55	5	25	2	10

a, Media comprised MS medium supplemented, as indicated by the medium designation, with either no hormones (MS), 6-BA at 1 mg/l (B1, NB, NBA), 6-BA at 2 mg/l (B2), 6-BA at 5 mg/l (B5), NAA at 0.02 mg/l (NB, NBA), adenine at 20 mg/l (NBA), or zeatin at 1 mg/l (Z1), 2 mg/l (Z2) or 5 mg/l (Z5).

nt, not tested

Table 3-2. The effect of transfer to various culture media on continued growth of callus derived from L. marginale protoplasts and subsequent shoot regeneration.

Medium[a]	No. of calli cultured	Calli which continued to grow		Growing calli which produced shoots	
		No.	%	No.	%
B1	120	0	0	–	–
NB	128	13	10.1	1	7.7
NBA	224	76	33.4	1	2.6
Z1	53	20	37.7	1	5
Z5	90	16	17.8	4	25
NZ	153	104	67.9	1	1.0
NZA	223	173	77.5	5	2.9

a, Media comprised MS medium supplemented, as indicated by the medium designation, with either 6-BA at 1 mg/l (B1, NB, NBA), NAA at 0.02 mg/l (NB, NBA, NZ, NZA), adenine at 20 mg/l (NBA, NZA), or zeatin at 1 mg/l (Z1, NZ, NZA) or 5 mg/l (Z5).

Results

Hypocotyls

After 7 days of culture, bud regeneration was observed along the length of hypocotyl explants, but with higher frequency near the cut ends (Fig. 3-1A). Many buds arose directly from the surface of the explants without forming any callus, while some arose on nodules which developed from the explants. Bud regeneration occurred on almost all media, even in the absence of added phytohormones (Table 3-1). The addition of cytokinin or cytokinin plus adenine in conjunction with auxin stimulated bud formation, not only by increasing the frequency of sites of bud initiation, but also by promoting the formation of clusters of buds at these sites. However, high concentrations of cytokinin (2 - 5 mg/l) inhibited bud initiation (Table 3-1) and growth. After 10 - 15 days of culture, small amounts of callus had developed at the cut ends of the explants, but few buds arose from this callus.

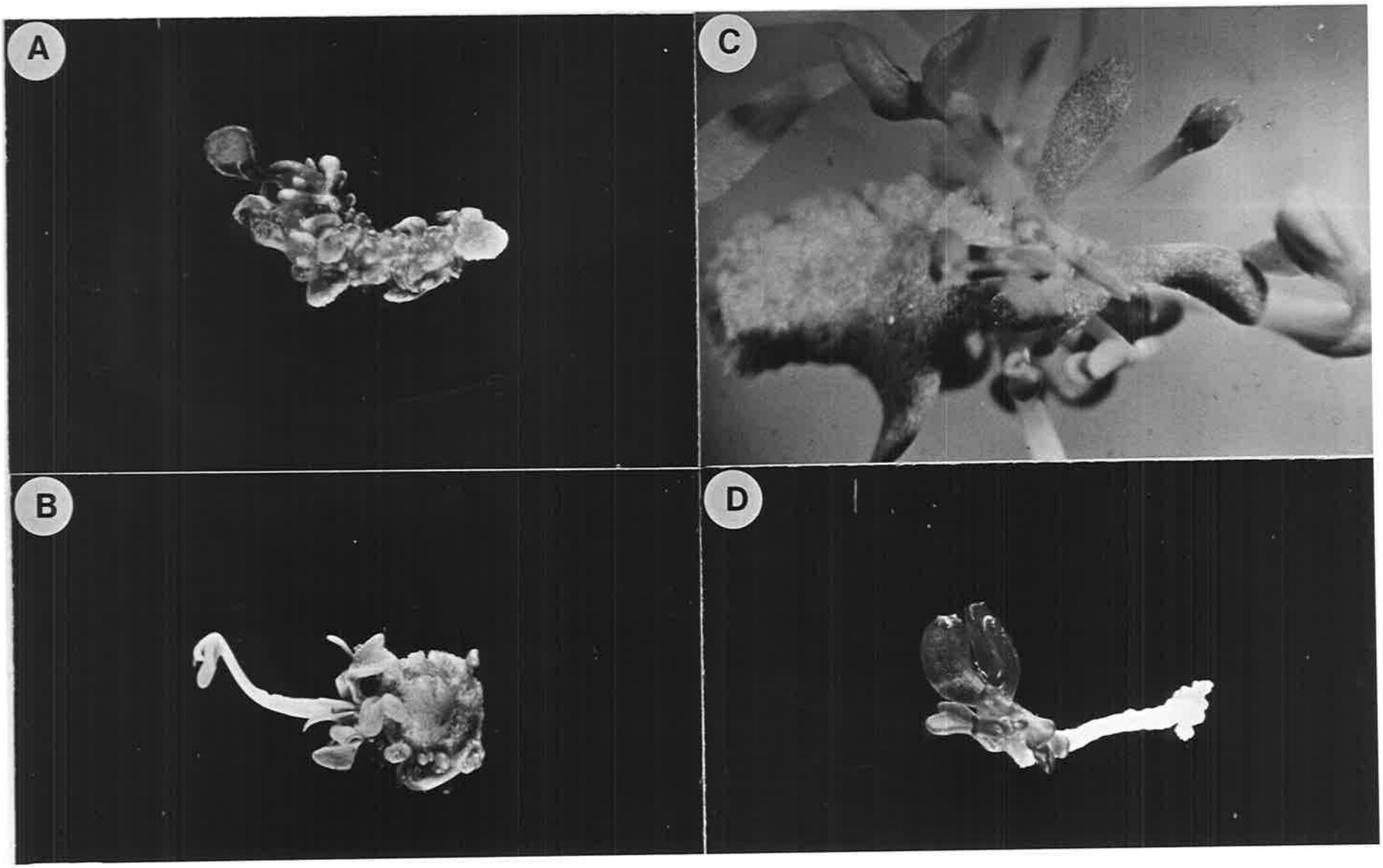
Cotyledons

After 10 - 15 days of culture, cotyledon explants had swollen a little, developed callus from the cut ends and nodules on their surface. Buds appeared after 16 days of culture on various media (Table 3-1). Most buds arose directly from the surface of the explants, some from nodules and a few from the cut ends (Fig. 3-1B). Both 6-BA and zeatin stimulated bud initiation, but 6-BA was better than zeatin (Table 3-1). However, fewer buds were initiated at higher concentrations of 6-BA. Adenine added together with 6-BA and NAA stimulated greater bud initiation (Table 3-1)

Leaves

After 10 days of culture, leaf explants started swelling, and by 20 - 25 days they had callused throughout. Multiple buds were regenerated from the callus after 25 days of culture (Fig. 3-1C) and medium Z1 was better than Z5 for bud regeneration (Table 3-1).

Fig. 3-1. Bud regeneration from explants of varous sources of L. marginale. A. From hypocotyl. B. From cotyledon. C. From leaf. D. From root.


A
C
B
D

Roots

After 10 - 20 days of culture, root explants had swollen and callused throughout. On B1, B2 and B5 media (Table 3-1) the explants turned yellow, and on the remainder they gradually turned green. Buds formed after 25 days of culture, but only on media containing zeatin (Table 3-1). In most cases, buds occurred on the upper side of the explant (Fig. 3-1D).

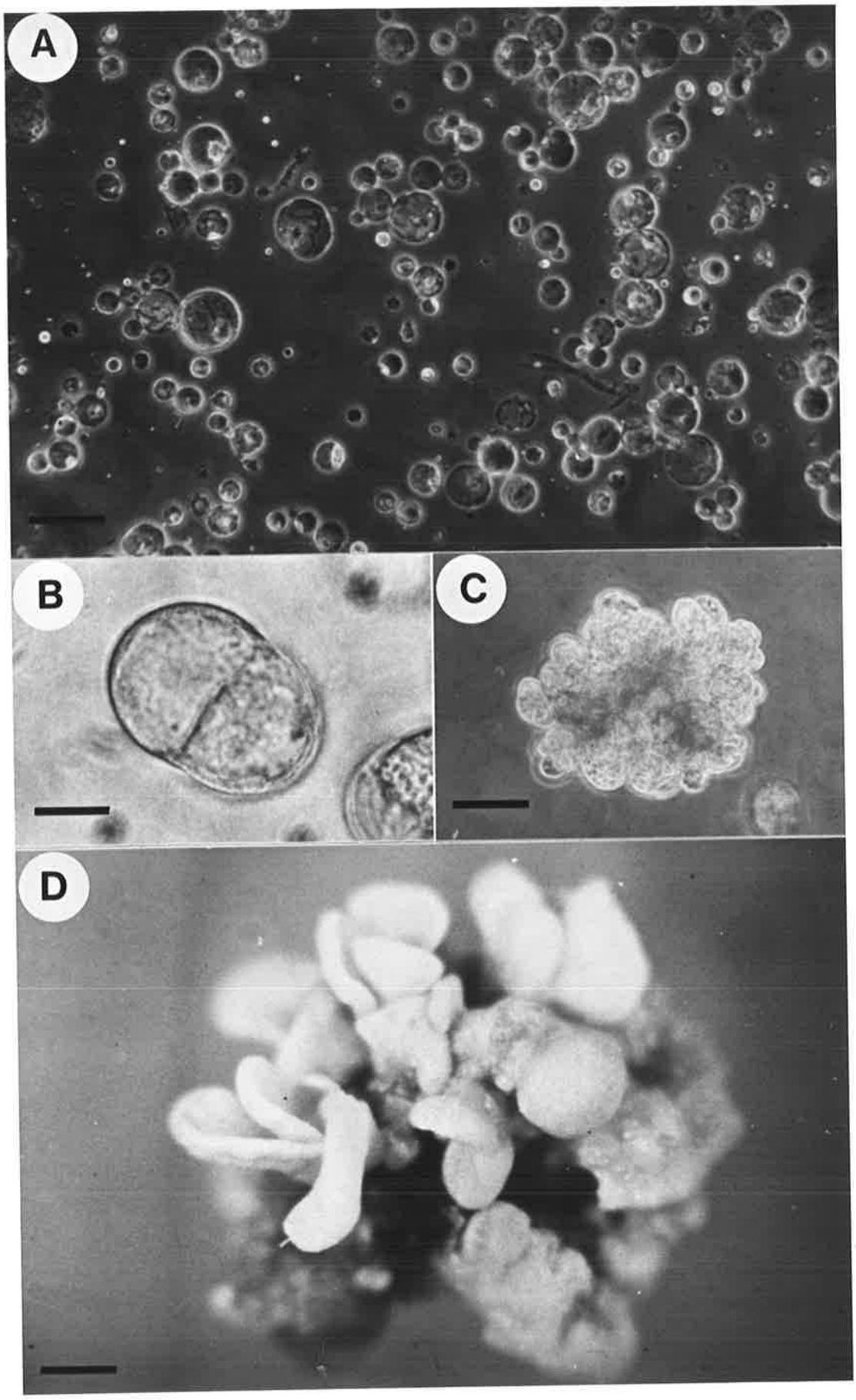
Protoplasts

Protoplasts were isolated readily from shoots (Fig. 3-2A), but with difficulty from roots and cotyledons. Dark treatment of shoot cultures before isolation of protoplasts had no significant effect on protoplast yield. Shoots, roots and cotyledons yielded 10^6, 1.5×10^4 and 8.5×10^3 protoplasts per gram fresh weight, respectively. Protoplasts isolated from each of the various explants looked healthy, but no divisions were found in protoplasts from roots and cotyledons. First divisions of protoplasts from shoots were observed within 48 h (Fig. 3-2B) and 15.3 ± 1.5 % of the protoplasts had undergone division after 1 week. The resulting calli (Fig. 3-2C) grew to 0.1 - 0.2 cm in diameter within 1 month and the calli continued growing after transfer to reach 0.5 - 0.8 cm in another month. Upon transfer to regeneration media, 6-BA alone was found to be insufficient to maintain callus growth. However callus growth was possible when zeatin alone was used (Table 3-2). NAA and adenine in addition to 6-BA or zeatin greatly enhanced callus growth (Table 3-2). Buds arose from callus about 1 month after transfer (Fig. 3-2D) and the medium with 5 mg/l zeatin was the best for bud regeneration (Table 2).

Regeneration of Rooted Plantlets

About 10% of shoots from each of the various sources produced roots to regenerate rooted plantlets.

Fig. 3-2. Bud regeneration from protoplasts of L. marginale. A. Protoplasts newly isolated from shoots (1cm = 36μm). B. First division of regenerated cells (1cm = 11μm). C. Compact protoplast - derived colony (1cm = 36μm). D. Buds regenerated from callus derived from protoplasts (1cm = 0.1cm).


A
B
C
D

Discussion

In vitro shoot regeneration of Linum marginale was satisfactorily achieved from various explants and from shoot protoplasts. Similar to L. usitatissimum, two types of bud regeneration were observed, either direct from the surface of explants or via callus.

Direct bud regeneration occurred very rapidly and efficiently on the surface of hypocotyl explants, in a manner similar to that for L. usitatissimum, in which buds arise directly from epidermal cells of the hypocotyl (Link and Eggers 1946). This kind of regeneration would be a good means of clonally multiplying L. marginale and may also be useful for genetic transformation techniques employing a DNA particle gun (Klein et al. 1987). The phenomenon of direct bud formation from hypocotyls is rare in most plant species, but occurs in at least two species of the genus Linum (this work; Link and Eggers 1946) and may be a feature of the genus worth further investigation.

Direct bud regeneration also occurred on cotyledon explants of L. marginale, although less efficiently than on hypocotyl explants. It does not occur at all in L. usitatissimum (Chapter 2).

Regeneration of buds occurred on callus derived from root and leaf explants and shoot protoplasts, but only root callus formed buds with high efficiency. It was interesting that roots (and possibly leaves) responded quite differently to the two cytokinins used in these experiments, compared to cotyledons. Roots responded to zeatin, but not to 6-BA, whereas cotyledons responded to 6-BA, but not to zeatin, except at high concentration (Table 3-1). Furthermore, roots and leaves responded by widespread conversion of tissue to callus prior to bud formation, but cotyledons by direct bud regeneration. This dichotomy may be worth further investigation. Widespread conversion of tissue to callus followed by regeneration of shoots has also been reported for roots of L. usitatissimum (Chapter 2; Zhan et al. 1989), but does not occur for leaves with this species.

Similar to L. usitatissium (Chapter 2), roots of L. marginale provide a possible system for transformation experiments using A. rhizogenes. The ability of L. marginale to regenerate shoots from protoplast-derived callus opens up several more options for genetic transformation of L. marginale, which include those discussed above, as well as microinjection (Morikawa and Yamada 1985; Crossway et al., 1986), electroporation (Shillito et al., 1985; Fromm et al., 1986), direct DNA uptake and polyethylene glycol induced uptake (Cocking et al., 1981; Krens et al., 1982; Paszkowsiki et al., 1984).

Clearly, there are a number of promising options for the genetic transformation of L. marginale which can be followed up. However, the efficiency with which shoots of L. marginale produce roots would need to be improved to enable efficient recovery of genetically transformed plantlets.

CHATER 4 REGENERATION OF FLAX FOLLOWING TRANSFORMATION BY AGROBACTERIUM

INTRODUCTION

Reecently, Basiran et al. (1987) reported the regeneration of transformed flax plants. By using an A. tumefaciens strain carrying a non-oncogenic npt-II gene and a wild type nopaline synthase gene, transformed shoots were regenerated from kanamycin resistant callus developed from inoculated hypocotyl sections. Jordan and McHughen (1988) also reported the regeneration of glyphosate tolerant flax plants by inoculating flax hypocotyl tissues with A. tumefaciens carrying a disarmed Ti - plasmid vector containing a chimeric npt-II gene and a glyphosate resistance plant-derived 5-enolpyruvylshikimate-3-phosphate synthase gene. Unfortunately, no data showing npt-II activity or the presence of T-DNA in transformed shoots are presented. In this chapter , the possibility of transforming flax with Agrobacterium was examined and regeneration of flax transformed by A. rhizogenes was achieved.

Materials and methods

Plant materials

Six cultivars of flax: Abyssinian, Akmolinsk, Bison, Bombay, Precederia and Stewart were used. Seeds of these cultivars were surface disinfected (see Chapter 2), then germinated and seedlings grown on hormone-free MS medium at 25 ^{0}C under a 16 h light (500 lux) / 8 h dark cycle.

Bacterial strains and plasmids

The Agrobacterium and Escherichia coli strains and plasmids used in this work are listed in Table 4-1. Agrobacterium strains were grown at 25 ^{0}C in Luria broth (LB) (Miller, 1972) without salt. E. coli strains were grown at 37 ^{0}C in LB containing 40 µg/ml ampicillin.

Table 4-1. Bacterial strains and plasmids

Species	Strains	Relevant plasmids	Opines encoded	Other relevant characters	Source	References
A. rhizogenes	A4	pRiA4	agropine and deoxy-mannityl-glutamine[a]	non-polar[b]	L. Moore	White and Nester, 1980a
	1855	pRi1855	agropine and deoxy-mannityl-glutamine	non-polar	J. Deley	Spanò et al., 1982
	TR7	pRiTR7	deoxy-mannityl-glutamine	polar	ICPB[c]	Petit et al., 1983
A. tumefaciens	C58C1	pGV3850:: pLGV2103	nopaline	chimeric npt-II gene and pBR322 sequences in T-DNA	P. Zambryski	Hain et al., 1985
E. coli	HB101	pMP27	-	EcoR1 fragments of pRi 1855 cloned in pBR322	P. Costantino	Pomponi et al., 1983
	HB101	pMP66	-		P. Costantino	Pomponi et al., 1983
		pBR322	-	-	BRESA[d]	Bolivar et al., 1977

a Agropine is encoded by the TR-DNA and deoxy-mannityl-glutamine by the TL-DNA of the Ri plasmid

b Non-polar strains of A. rhizogenes contain TL and TR-DNA on their Ri plasmids whereas polar strains only carry TL-DNA. TR-DNA encodes auxin biosynthesis, which confers auxin independence to the root induction process i.e. roots are induced independently of the polarity of auxin transport in an inoculated explant (Ryder et al., 1985).

c ICPB = International Collection of Phytopathogenic Bacteria

d BRESA = Biotechnology Research Enterprises South Australia

Transformation and regeneration using *A. tumefaciens*

To transform and regenerate flax using A. tumefaciens, the strain C58C1 carrying the disarmed Ti plasmid pGV3850::pLGV2103 was used (Hain et al., 1985). This plasmid was derived from pTiC58 and contains between the T-DNA borders; a chimeric npt-II gene, which was used as a selective marker conferring kanamycin resistance to transformed plant tissue; the nopaline synthase gene, which was used as a non-selective marker; and pBR322 sequences, which were used as targets in Southern hybridization analysis.

The approach to the use of hypocotyls was based on two observations, first, that regeneration of shoots from hypocotyls is very efficient (Link and Eggers, 1946; Gamborg and Shyluk, 1976; Mathews and Narayanaswamy, 1976; Chapter 2), and secondly, that most of these shoots arise directly from epidermal cells of the hypocotyl (Link and Eggers, 1946). The main strategy was therefore to attempt to transform epidermal cells of the hypocotyl without altering their capacity for direct regeneration.

Hypocotyl sections, 0.8–1 cm in length, from 7-day-old seedlings of Bison, Stewart and Akmolinsk were inoculated at room temperature with an overnight liquid culture of A. tumefaciens that had been incubated either with, or without, 100–200 μM sinapinic acid. This compound specifically activates the Vir region of the Ti plasmid (Stachel et al., 1985) and was used in an attempt to maximize transformation efficiency and as a possible means of effecting the transformation of epidermal cells even if they were not wounded.

Several different methods of inoculation were employed: 1) explants were submerged and shaken in the bacterial culture at 50 r.p.m. for 1–2 hours, 2) explants were gently rubbed with a mixture of the bacterial culture and an abrasive powder (celite), to remove the cutin layer of the hypocotyl and injure epidermal cells, 3) explants were placed in the bacterial culture, then vacuum infiltrated at -85 kPa for 3–5 minutes in an attempt to stress epidermal cells and to drive bacteria into the stomata, so that bacteria might gain access to epidermal cells from within stomata.

After inoculation, the explants were blotted dry. Half the explants for each treatment were transferred to MS medium containing 0.02 mg/l napthalene acetic acid (NAA), 1 mg/l 6-benzylaminopurine (6-BA) and 20 mg/l adenine (NBA medium) and the other half were transferred to NBA medium containing 200 μM sinapinic acid. All explants were incubated at 25 ^{0}C under a 16 hour light (1000 lux) / 8 hour dark cycle for 2 days. Then the explants were transferred to NBA medium containing 400–500 μg/ml cefotaxime and 100 μg/ml kanamycin (NBACfKm medium) and incubated as above. Green buds that formed on the explants were cut off when they reached about 1 cm in length and placed on MS medium containing 400–500 μg/ml cefotaxime and 100 μg/ml kanamycin (CfKm medium) to allow root formation. Several thousand hypocotyl explants were used in these experiments.

Cotyledon explants, 0.5 cm x 0.5 cm, from 7-day-old seedlings of Akmolinsk and Precederia were submerged in an overnight liquid-culture of A. tumefaciens for 1–2 hours. After blotting dry, they were transferred to MS medium containing 1–2 mg/l 6-BA and incubated as above for 2 days. Then, explants were transferred to MS medium containing 1–2 mg/l 6-BA, 500 μg/ml cefotaxime and 100 μg/ml kanamycin (BCfKm medium), and incubated as above. About 30 days later, calli developing from the cut edges of each piece were cut into small pieces (2–3 mm in diameter), and put on BCfKm medium again for regeneration. About a thousand cotyledon explants were used in these experiments.

Transformation and regeneration using *A. rhizogenes*

Cotyledons from 7-day-old seedlings of Abyssinian, Akmolinsk, Bombay, Precederia were detached, cut into halves, and submerged in an overnight liquid culture of A. rhizogenes for about 2 hours. Then they were blotted dry, transferred to MS medium, and incubated as above for 2 days, except that diffuse light was used during the light phase of the cycle. They were then transferred to MS medium containing 500 μg/ml cefotaxime and incubated as above with diffuse light. Roots, 0.5–1 cm in length, which developed at the cut ends of cotyledons, were excised and

placed on NBA medium containing 500 µg/ml cefotaxime (NBACf medium) and incubated as above (with strong light) to allow shoot formation.

DNA extraction

Total plant DNA was extracted using a method derived from that of Rogers and Bendich (1985), as follows: Tissue was frozen in liquid nitrogen and pulverized with a mortar and pestle. One ml of 2x CTAB extraction buffer (2% [w/v] cetyl-trimethyl-ammonium bromide, 100 mM Tris pH 8.0, 20 mM disodium EDTA pH 8.0, 1.4 M NaCl) was added to each gram of tissue. The slurry was incubated at 65 ^{0}C for 5 minutes. An equal volume of chloroform was added and the suspension was gently but thoroughly mixed. Then the emulsion was centrifuged at 11 000 g at 4 ^{0}C for 5 minutes. The upper phase was transferred to another tube, one volume of 1% CTAB was added, and the suspension gently mixed, then incubated at room temperature for 30 minutes. The suspension was then centrifuged as above. The pellet was washed once with 0.1 M sodium acetate in 75% ethanol, once with ice cold 75% ethanol, dried briefly in air, and dissolved in 0.1x TE buffer (1 mM Tris, 0.1 mM EDTA, pH 8.0).

Plasmid DNA was prepared by alkaline lysis and purified by cesium chloride density gradient centrifugation in the presence of ethidium bromide as described by Maniatis et al. (1982).

Gel electrophoresis, Southern transfer and DNA hybridizations

Plasmid and plant DNA were digested to completion with HindIII (Pharmacia) according to the manufacturer's instructions, then separated by electrophoresis in a 0.8% agarose gel as described by Maniatis et al. (1982), and transferred to a Gene Screen Plus membrane (Du Pont) according to the manufacturer's instructions. Probe plasmids were labeled with ^{32}P-labeled dCTP using a nick translation kit (BRESA) and hybridization carried out as described by Maniatis et al. (1982). Since pRi1855 is almost identical to pRiA4 (Jouanin, 1984), pMP27 and pMP66, which contain most of the TR and TL-regions of pRi1855, respectively (Pomponi et al., 1983), were used as

probes in Southern hybridization analysis of plantlets transformed by both 1855 and A4. pBR322 (Bolivar et al.,1977) was used as a probe in Southern hybridization analysis of plant tissues transformed by A. tumefaciens carrying pGV3850::pLGV2103.

Opine assays

Opine analysis by high voltage paper electrophoresis was carried out as described by Yamada and Itano (1966) to detect nopaline, and by Ryder et al. (1985) to detect agropine and deoxy-mannityl-glutamine.

Results

Transformation and regeneration using *A. tumefaciens*

Ten to 15 days after inoculation, callus appeared on the cut ends of inoculated hypocotyl and cotyledon explants in all treatments on media containing kanamycin. These calli continued growing vigorously on media containing 100 μg/ml kanamycin. Large amounts of nopaline were detected in these calli and Southern hybridization analysis showed they were transformed (data not shown). In a few cases, buds appeared on kanamycin resistant callus, but they all remained small and failed to develop on medium containing kanamycin.

Seven to 10 days after inoculation many buds appeared along the surface of hypocotyl segments cultured on NBACfKm medium. Most buds were white and of the few green buds that did appear most bleached out gradually. In contrast, only a few white buds appeared on uninoculated hypocotyl segments on the same medium. After about 4 weeks, some buds on inoculated hypocotyl sections, including both white and green buds, had grown to 1–2 cm in length. Small amounts of nopaline were detected in both white and green shoots (data not shown).

Green shoot tips about 1 cm in length were excised and transferred to CfKm medium where some of them developed very short roots. However, when they were

repeatedly subcultured on the same medium, nopaline could no longer be detected (data not shown), the shoots became bleached and no roots formed. Southern hybridization analysis of DNA from two of the shoots which initially produced short roots showed that they were not transformed (data not shown). In all, about 100 green buds were regenerated on inoculated hypocotyl sections, but none was transformed.

The attempt to injure epidermal cells by rubbing with an abrasive powder may have assisted transformation, but it also stimulated callus formation and suppressed shoot regeneration. No transformed shoots were regenerated from epidermal cells around vacuum infiltrated stomata, and the addition of sinapinic acid had no noticeable effect.

Transformation and regeneration using *A. rhizogenes*

About 20 days after inoculation, numerous adventitious roots appeared at the cut end of almost all cotyledon explants inoculated with strains 1855 and A4, but those inoculated with TR7 produced far fewer roots. Buds regenerated on roots 20–35 days after they were excised and transferred to NBACf medium. Roots induced by *A. rhizogenes* showed a much lower frequency of shoot regeneration compared to untransformed roots (Table 4-2). For the cultivar Bombay, the differences in bud regeneration between untransformed roots and those induced by 1855, A4 or TR7 were all statistically significant ($\chi_1^2 = 11.65$, $p < 0.01$; $\chi_1^2 = 9.39$, $p < 0.01$; and $\chi_1^2 = 6.22$, $0.01 < p < 0.02$, respectively). Furthermore, the data for the two non-polar strains , 1855 and A4, were homogeneous ($\chi_1^2 = 0.16$, $0.5 < p < 0.7$). These data were pooled and compared with those for the polar strain TR7, by Fisher's exact test, and no significant difference was found ($p = 0.26$).

Analysis of plants transformed by *A. rhizogenes*

Opine analysis showed that 7 regenerated plants were opine positive (data not shown). Four of these were chosen for further study. These comprised Pre-1 and Bom-5, both transformed by 1855 and derived from Precederia and Bombay,

Table 4-2. Number of A. rhizogenes-induced roots that formed buds and the number of buds that produced opine

Flax cultivar	A. rhizogenes strains or hormone	Number of roots placed on NBACf medium	Number of roots forming buds	Percentage of roots forming buds	Number of buds producing opine	percentage of buds producing opine
Akmolinsk	1855	300	1	0.33%	-	-
Abyssinian	1855	420	0	-	-	-
Precederia	1855	240	1	0.42%	-	-
Bombay	1855	98	5	5.1%	2	40.0%
	A4	192	12	6.25%	2	16.7%
	TR7	48	2	4.17%	2	100.0%
Bombay	NAA 0.5 mg/l	80	16	20.0%	-	-

respectively, and Bom-1 and Bom-3, both transformed by A4 and derived from Bombay. Bom-1, Bom-3 and Bom-5 lost their ability to synthesize opines after several subcultures (Fig.4-1), despite the presence of T-DNA (Fig. 4-2), whereas Pre-1 retained this ability.

Southern hybridization analysis showed hybridizing bands co-migrating with HindIII restriction fragments 19, 23, 37b and 45 of pMP66 present in DNA from plantlets Pre-1, Bom-3 and Bom-5, and similarly for Bom-1 with the exception of fragment 19, which was missing (Fig. 4-2A). Additional hybridization bands of various fragment sizes and intensities, that did not co-migrate with any of the restriction fragments in pMP66 were also evident (Fig.4-2A). One of these corresponds to internal TL-DNA restriction fragment 38 which is not wholly contained within pMP66 (Fig. 4-2C). The others presumably represent junction fragments containing TL-DNA joined to flax DNA or to itself in tandem repeats. Since pMP66 does not include the right side of the TL-region of pRi1855 (Fig. 4-2C), then only junction fragments involving the left side of the TL-DNA would have been detected. From the multiplicity of these fragments, it would appear that multiple copies of the TL-DNA may have been integrated into Pre-1 and Bom-3.

An intense hybridizing band co-migrating with HindIII restriction fragment 16b in pMP27 was present in DNA from Pre-1. Bands co-migrating with fragment 24 and another small fragment in pMP27 were also present in DNA from Pre-1, but the hybridization was much less intense compared to the band co-migrating with 16b. Several other hybridization bands of various fragment sizes and intensities were also detected, but none of these appeared to correspond to HindIII restriction fragments of pMP27 (Fig.4-2B). Since pMP27 includes both sides of the TR-DNA of pRi1855 (Fig. 4-2C), these bands presumably represent junction fragments containing both sides of the TR-DNA joined to flax DNA or themselves in tandem repeats. From the multiplicity of these fragments, it would also appear that multiple copies of the TR-DNA may have been integrated into Pre-1. There was no evidence for insertion of TR-DNA into the DNA of Bom-1, Bom-3, or Bom-5 (Fig. 4-2B).

Fig. 4-1. Opine assays of plants regenerated from hairy roots and subcultured on MS medium containing 500 µg/ml cefotaxime; 2 µl of supernatant from 30 mg of tissue homogenized in 30 µl of 0.1 M Tris-HCl pH 8 were applied in each lane. Lane 1, opine mixture standard containing 10 µg agropine (a) and 10 µg deoxy-mannityl-glutamine (m); lane 2, extract from Bom-3: lane 3, extract from Bom-1; lane 4, extract from Bom-5; lane 5, extract from Pre-1. The migration of opines was retarded in plant extracts compared to the opine standards. o = the origin; Bom = Bombay; Pre = Precederia.

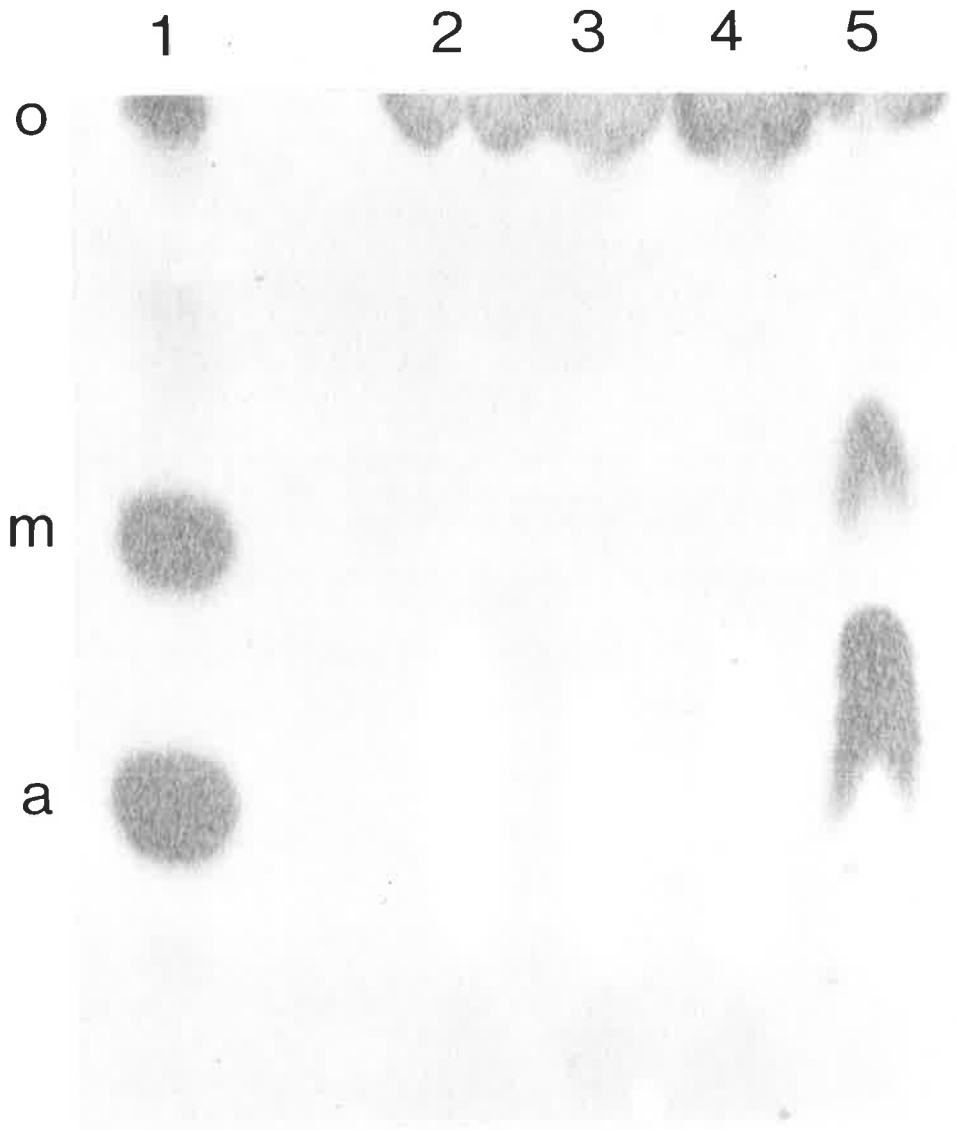
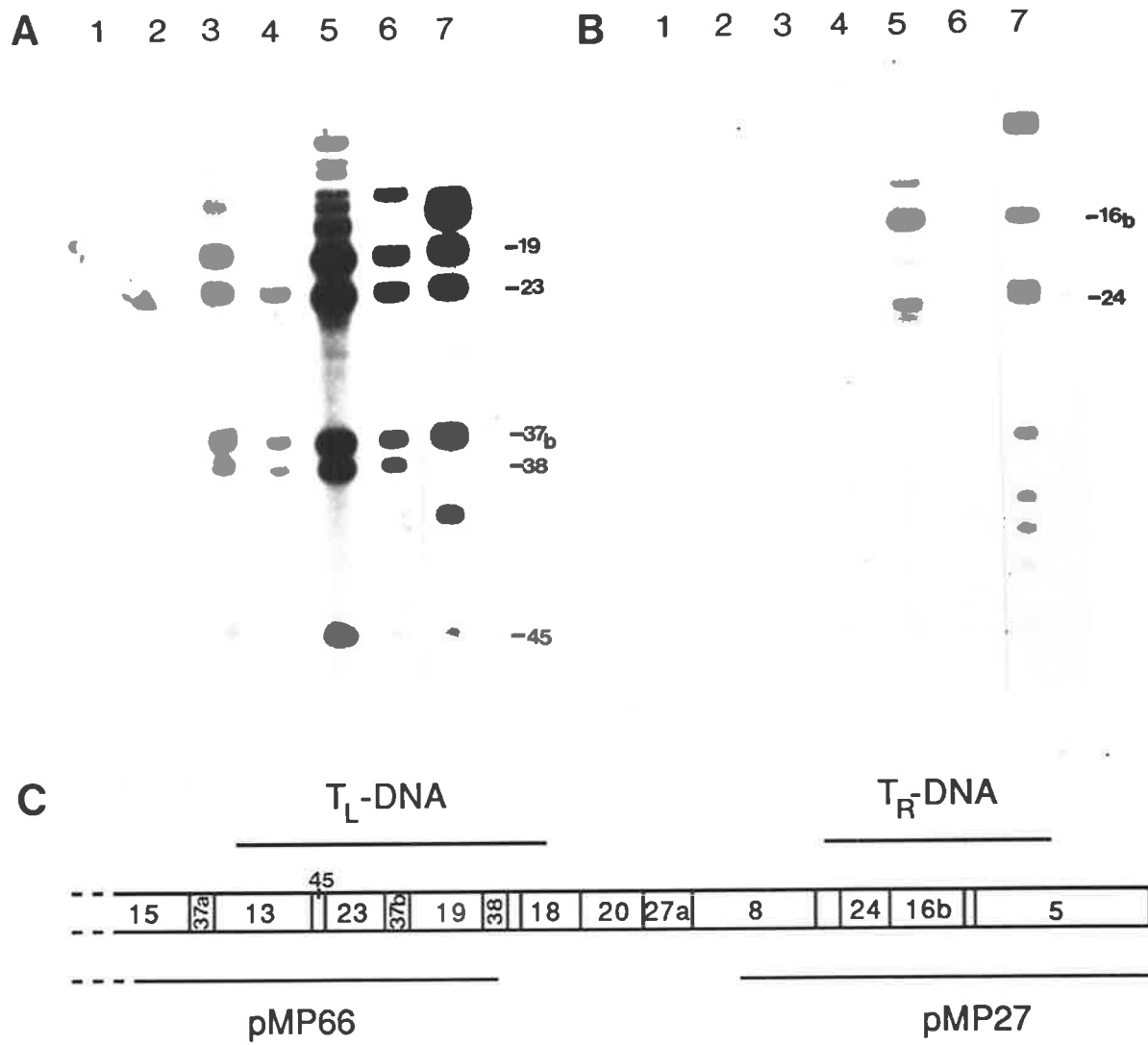

1
2
3
4
5
o
m
a

Fig. 4-2. A and B. Southern hybridization analysis of HindIII digested DNA from hairy root regenerants, Pre-1, Bom-1, Bom-3, Bom-5, and their untransformed progenitors, Precederia and Bombay. Approximately 15 μg of plant DNA was loaded in each lane. Lane 1, untransformed Bombay; lane 2, untransformed Precederia; lane 3 Bom-3; lane 4, Bom-1; lane 5, Pre-1; lane 6, Bom-5; lane 7, 1–2 copy reconstruction using HindIII digested probe DNA. A. Probed with pMP66. B. Probed with pMP27. The numbers on the right correspond to restriction fragments in C. The arrow in B indicates the position of a small fragment in lane 5 which co-migrates with a small fragment of HindIII digested pMP27 in lane 7. C. HindIII restriction map of the T-DNA regions of pRi1855 based on the map of Spanò et al. (1987).


A
1 2 3 4 5 6 7
-19
-23
-37b
-38
-45
B
1 2 3 4 5 6 7
-16b
-24
C
T_L-DNA
T_R-DNA
45
15
37a
13
23
37b
19
38
18
20
27a
8
24
16b
5
pMP66
pMP27

These data indicate that one of the four plantlets, Pre-1, was transformed by both TL-DNA and TR-DNA and that the other three were transformed only by TL-DNA, and in the case of Bom-1 by only part of the TL-DNA.

Morphology of plants transformed by A. rhizogenes

Transformed plants grown in vitro exhibited curled leaves, short internodes, and some had a more developed root system characterized by plagiotropic behaviour, compared to normal regenerants (Fig. 4-3). In at least two cases some side shoots recovered normal morphology during subculture of transformed plants.

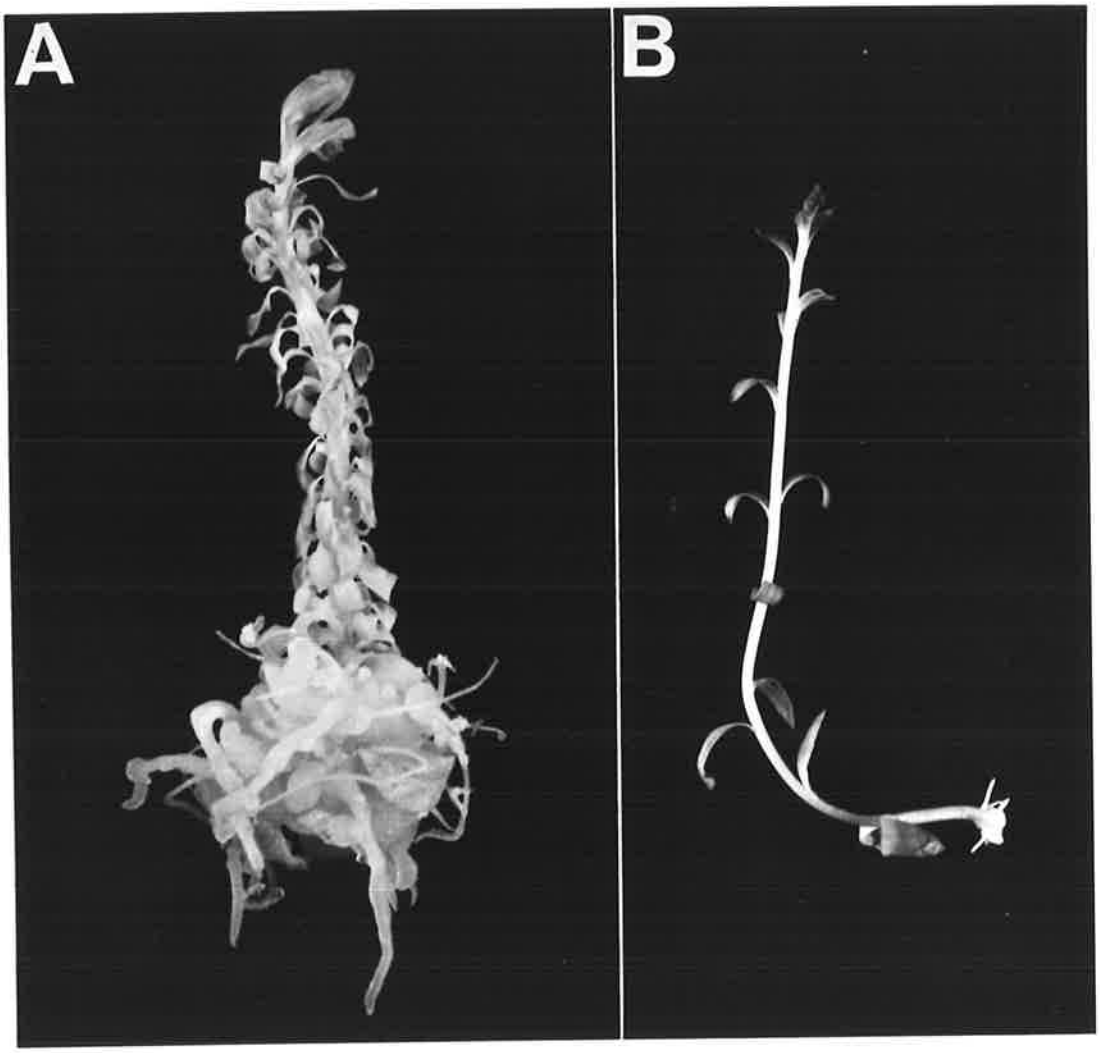
Discussion

Initially, it was hoped to regenerate transformed shoots from hypocotyl segments inoculated with A. tumefaciens containing a disarmed Ti-plasmid, because direct regeneration of buds from the epidermal cell layer of hypocotyls is very efficient (Link and Eggers, 1946; Gamborg and Shyluk, 1976; Mathews and Narayanaswamy; 1976). However, this was not achieved. The differentiation on inoculated hypocotyls of buds which initially appeared green in the presence of kanamycin was presumably due to inefficient kanamycin selection, perhaps allowing transformed callus to cross protect untransformed shoots. The nopaline detected in these shoots presumably diffused from the transformed callus. Similar problems with the occurrence of false positives have also been reported for attempts to regenerate transformed plants from cotton hypocotyls inoculated with A. tumefaciens (Firoozabady et al., 1987).

Also, no transformed shoot from callus on inoculated hypocotyls or cotyledons was obtained, presumably because the regeneration rate of transformed callus was too low in the cultivars that used. However, it should be possible, although probably difficult, to obtain transformed plants by this means.

In contrast to the results for A. tumefaciens, transformed plants were obtained readily from hairy roots induced by A. rhizogenes. This would appear to be the first

Fig. 4-3. A. Transformed flax plantlet Bom-5 with short internodes, prolific root system and curled leaves. B. Untransformed flax plantlet of the cultivar Bombay.


A
B

report of the regeneration of flax transformed by A. rhizogenes. These results show that transformation by A. rhizogenes is an effective alternative to transformation by disarmed strains of A. tumefaciens for the genetic engineering of plants, especially when it is not possible to regenerate shoots readily from callus but is possible from roots.

In transformed plants obtained from hairy roots, the spontaneous disappearance of opines and the recovery of normal plant morphology without the loss of T-DNA has also been reported previously (Tepfer, 1984), and is perhaps due to cytosine methylation of inserted T-DNA (Hepburn et al., 1983). Considering this fact, together with the fact that opines diffuse from transformed to untransformed tissue, the opine assay is not always a reliable measure of transformation, since tissue without opines may be transformed, while tissue with small amounts of opine may not be transformed. So, the use of opine assays alone to judge whether a tissue or plant is transformed must be treated with caution.

These results also show that the frequency of buds regenerated from roots induced by either polar or non-polar strains of A. rhizogenes was significantly lower than that for untransformed roots. Since the Ri plasmid in polar strains contains only TL-DNA, one interpretation is that expression of TL-DNA in roots interferes with shoot regeneration. In addition, Southern hybridization analysis showed that three of the four transformed plants analysed did not have insertions of TR-DNA, although all four were transformed by non-polar strains of A. rhizogenes. Furthermore, in Pre-1 the hybridization intensity for the left part of the TR-DNA, viz. the band corresponding to HindIII restriction fragment 24 in pMP27, was much lower than that for the right part, viz. the band corresponding to fragment 16b in pMP27 (Fig. 4-2B). This suggests that deletions or re-arrangements of the left part of the TR-DNA may have occurred, leading to the left part of the TR-DNA, which contains aux-2, an auxin biosynthesis gene (Spanò et al.,1987), being under-represented. These data may be consistent with the suggestion made by Spanò et al. (1987) that expression of aux-2 in

transformed roots hinders bud regeneration, so that only roots or root tissue deficient in TR-DNA regenerate shoots.

CHAPTER 5 THE pTiC58 TZS GENE PROMOTES HIGH EFFICIENCY ROOT INDUCTION BY AGROPINE STRAIN 1855 OF AGROBACTERIUM RHIZOGENES

Introduction

Two phytohormone genes have been found outside the T-DNA region. One is a cytokinin gene (tzs) which is responsible for trans-zeatin synthesis; it is found in the Vir region of nopaline Ti-plasmids and in agropine and mannopine Ri-plasmids, but not in octopine Ti-plasmids nor the agropine Ti-plasmid, pTiBo542 (Beaty et al. 1986; Akiyoshi et al. 1987). The expression of tzs is regulated by the Vir region and like the vir genes is inducible by plant phenolics (Alt-Moerbe et al. 1988; John and Amasino, 1988; Powell et al., 1988), although only very low levels of trans-zeatin are produced by strains of A. rhizogenes carrying agropine Ri-plasmids (Akiyoshi, 1987).

The second is an auxin gene (iaaP) which is also found in the Vir region of the nopaline Ti-plasmid, pTiC58, but, unlike the vir genes, it is constitutively expressed (Liu et al., 1982). Both genes seem to play a role in plant cell transformation by the T-DNA of pTiC58. The iaaP gene is suggested to be essential for transformation (Liu et al.,1982), and the tzs gene, although not essential, may promote transformation (Alt-Moerbe et al.,1988; John and Amasino, 1988). In this chapter, data are presented supporting the latter proposition, through the novel use of the tzs gene to markedly increase the induction of hairy roots by an agropine strain of A. rhizogenes.

Materials and Methods

Plant Material

Seeds of the flax cultivar Akmolinsk were surface disinfected for 30 min with 3% sodium hypochlorite containing 0.1% Tween 80, washed several times with sterile distilled water, then germinated and grown on hormone free Murashige and Skoog (MS) (1962) medium at 25 ^{0}C under a 16 h light (500 lux) / 8 h dark cycle.

Bacterial strains and plasmids

Bacterial strains and plasmids used are shown in Table 5 - 1.

Bacterial culture and matings

Agrobacterium was grown at 25 ^{0}C in Luria broth (LB) (Miller, 1972) without salt and E. coli was grown at 37 ^{0}C in LB. Antibiotics used were, for E.coli: ampicillin (40 µg/ml) and for Agrobacterium: rifampicin (100 µg/ml), kanamycin (100 µg/ml). Triparental matings (Ditta et al., 1980) were used to transfer pUCD1186 from E. coli to Agrobacterium strains C58C1 and LBA4404. One ml aliquots from overnight cultures of the donor, helper (RK2013) and recipient were mixed and filtered onto sterile 0.45 µm pore size Millipore filters which were incubated on non-selective LB agar plates overnight at 28 ^{0}C. Bacteria were suspended in 10 ml LB without salt, a tenfold dilution series prepared and 100 µl aliquots of appropriate dilutions plated on selective media. The plasmid profiles of donors, recipients and exconjugants were examined by electrophoresis of plasmid mini-preparations in 0.5% agarose gels, as described by Maniatis et al. (1982), and appropriate transconjugants selected. Mini-preparations of plasmids from E. coli and Agrobacterium were performed as described by Maniatis et al. (1982) and Farrand et al. (1985), respectively.

Transformation experiments

Fresh overnight liquid cultures of the various strains of A. tumefaciens (Table 5-1) and strain 1855 of A. rhizogenes were centrifuged at 4000 x g at 4 ^{0}C for 10 min and the cells resuspended in liquid YMB (Hooykaas et al., 1977) medium. The A. tumefaciens strains were mixed pairwise with A. rhizogenes to final densities of 0.5 x 10^8 cells/ml for A. tumefaciens and 5 x 10^8 cells/ml for A. rhizogenes. Cotyledons from seven day old seedlings of flax were cut into halves, submerged in either a suspension of A. rhizogenes at 5 x 10^8 cells/ml or in the bacterial mixtures for 2 h, blotted dry, transferred to MS medium and incubated at 20 ^{0}C in the dark for 2 days. After that, they were transferred to MS medium containing 500 µg/ml cefotaxime and

Table 5-1. Bacterial strains and plamids.

Species	Strains	Relevant plasmids	Other relevant characters	Source	References
A. rhizogenes	1855	pRi1855	non-polar agropine strain	J. DeLey	Spanò et al., 1982
A. tumefaciens	GV3850	pGV3850	C58C1 background onc$^-$,vir$^+$,iaap$^+$,tzs$^+$,rif^R,Cb^R	P. Zambryski	Hain et al., 1985
	C58C1	-	onc$^-$,vir$^-$,iaap$^-$,tzs$^-$,rif^R	J. Schell	Van Larebeke et al., 1974
	K1067	pUCD1186	C58C1 background onc$^-$,vir$^-$,iaap$^-$,tzs$^+$,rif^R,Cb^R,Km^R	this work	
	LBA4404	pAL4404	Ach5 background onc$^-$,vir$^+$,iaap$^-$,tzs$^-$,rif^R,Sm^R	J. Ellis	Ooms et al., 1982
	K1128	pAL4404 + pUCD1186	LBA4404 background onc$^-$,vir$^+$,iaap$^-$,tzs$^+$ rif^R,Sm^R,Cb^R,Km^R	this work	

Table 5-1. continued

E.coli	DH1	pUCD1186	cloned enzyme Bgl ll fragment of pTiC58 carrying tzs in the mobilizable vector pUCD615, Ap^R,Km^R	T. Close	Rogowsky et al., 1987
	RK2013	pRK2013	plasmid mobilizing strain	J. Ellis	Ditta et al., 1980
	K1004	pMP27	HB101 background,cloned EcoR1	P. Costantino	Pomponi et al., 1983
	K1005	pMP66	fragments of pRi1855 covering the TL- and TR-DNA, respectively	P. Costantino	

incubated at 25 ^{0}C under a 16 h diffuse light/8 h dark cycle. A proportion of the explants inoculated with A rhizogenes strain 1855 alone were incubated as above on media also containing 0.1 or 0.2 mg/l kinetin. At least two callus pieces from each of six independent root clones were cultured as above on MS medium containing 500 µg/ml cefotaxime, 0.5 mg/l IAA and 2 mg/l kinetin until large enough for DNA analysis.

DNA extraction and Southern hybridization

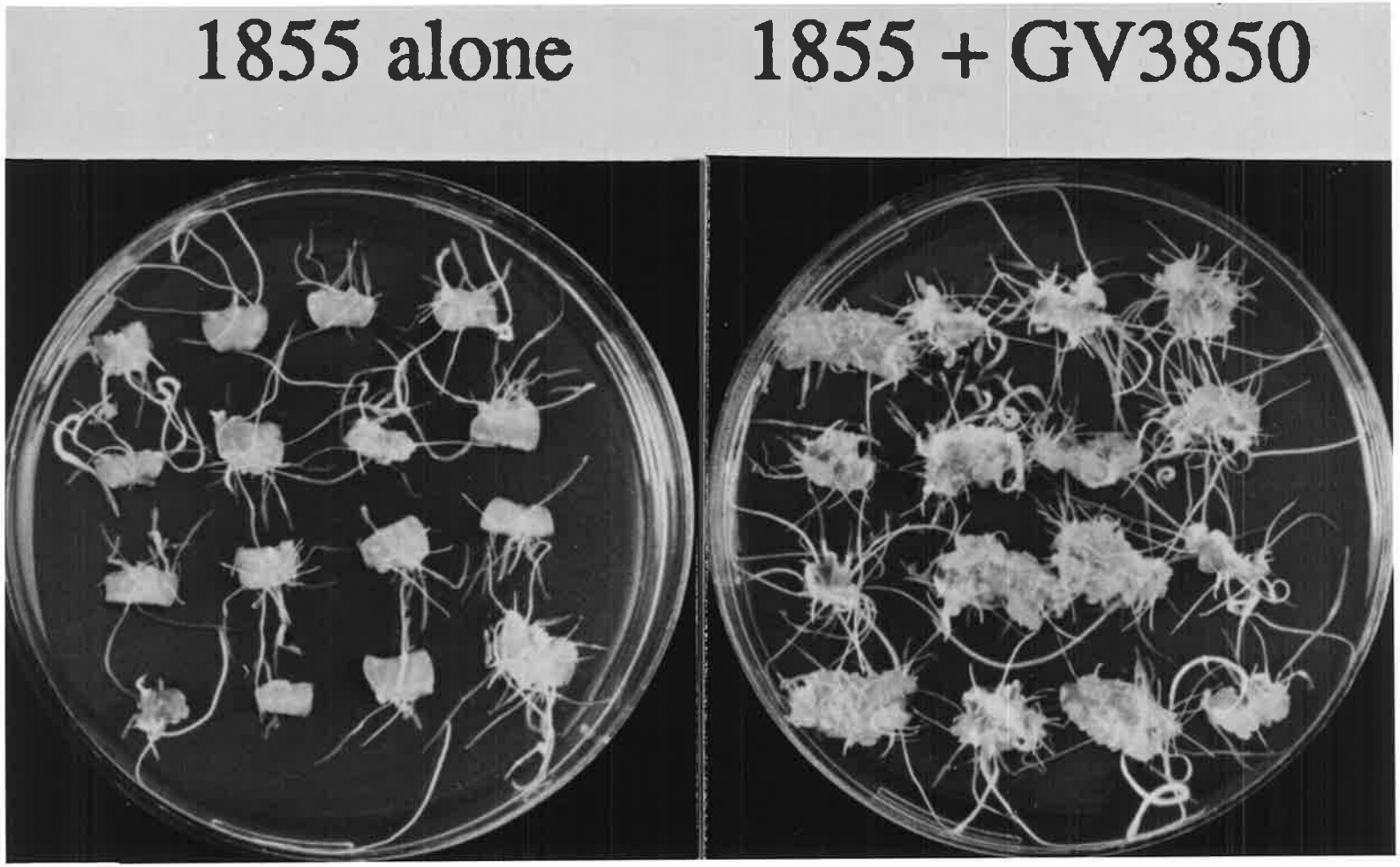
Plasmids pMP27 and pMP66, which contain most of the TR and TL regions of pRi1855 were mixed and used as probes of total DNA from calli derived from roots induced by mixed inoculation.Plasmids were prepared and purified by cesium chloride density gradient centrifugation according to Maniatis et al. (1982). Total DNA from root callus was prepared and examined by Southern hybridization according to Zhan et al. (1988 and Chapter 4)).

Results

Root induction

Roots began appearing at the cut ends of cotyledons in all treatments after 6-7 days of culture and differences between treatments were clear by 15-20 days. Initial experiment showed that there were many more roots, greater root vigour and more callus on explants inoculated with both A. rhizogenes strain 1855 and A. tumefaciens strain GV3850 than on those inoculated with 1855 alone (Fig. 5-1). GV3850 is a strain of C58C1 background containing pGV3850 which is a disarmed plasmid pTiC58 but still keeps nos gene, a gene and part of b gene. Also it contains the tzs gene and the iaaP gene in its Vir region. In order to identify which one promotes the root induction by strain 1855, a number of A. tumefaciens strains with different contents were used with 1855 in inoculation experiments. They were: C58C1, a cured strain of nopaline strain C58 for providing chromosome background of GV3850; LBA4404, a disarmed octopine strain Ach5 containing octopine type plasmid

Figure 5-1. The effect on root induction of inoculating flax cotyledon explants with A. rhizogenes strain 1855 alone or in combination with disarmed A. tumefaciens strain GV3850.


1855 alone
1855 + GV3850

pAL4404 which completely lacks T-DNA but contains the Vir region; K1067, a strain of C58C1 background containing plasmid pUCD1186 with tzs gene; K1128, a strain of Ach5 background containing plasmid pAL4404 and pUCD1186. All strains were used in mixed inoculations with 1855 on flax explants for root induction. Results showed that (Fig. 5-2) only when 1855 was mixed with K1128 in inoculation did high efficiency root formation occur indicating the Vir region and tzs gene in combination were involved in promoting root inductuion by 1855.

High efficiency root production could not be reproduced by providing 1855-inoculated explants with an exogenous source of cytokinin, since culturing them after inoculation on medium containing 0.1 or 0.2 mg/l kinetin produced no more roots than on medium without kinetin, although the roots were more vigorous and more callus was formed (data not shown).

Southern hybridization analysis

Six callus clones were analysed by Southern hybridization and five were found to have hybridizing bands comigrating with HindIII restriction fragments 19, 23, 37b, and 45b of the pRi1855 TL-DNA contained in pMP66 and to fragment 38 which is not wholly contained within pMP66 (Fig. 5-3). None had any bands clearly corresponding to HindIII restriction fragments of the pRi1855 TR-DNA contained in pMp27.

Discussion

In mixed inoculations, the results clearly show the strong promoting effect of disarmed strains of A. tumefaciens carrying an intact Vir region and a tzs gene on root induction by A. rhizogenes strain 1855. Alt-Moerbe et al. (1988), John and Amasino (1988) and Powell et al., (1988) have shown that the tzs gene is vir inducible and the secretion of zeatin was increased more than 100-fold by induction. It seems most likely that the root promoting effect is mediated by vir-induced trans-zeatin synthesis and secretion by the disarmed strains of A. tumefaciens.

Figure 5-2. The effect on root induction of co-inoculating flax cotyledon explants with A. rhizogenes strain 1855 and A. tumefaciens carrying various combinations of the Vir region and tzs.

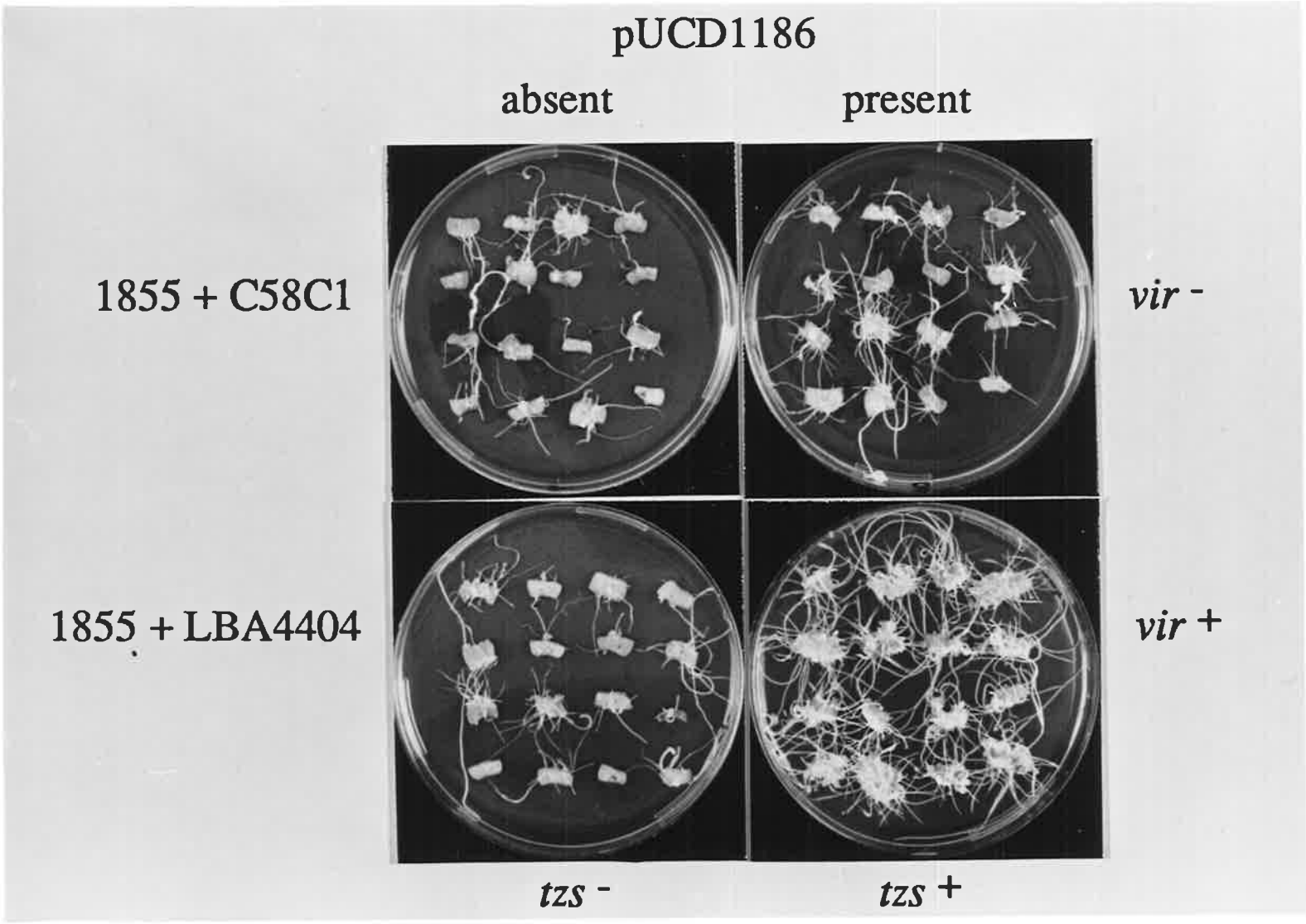
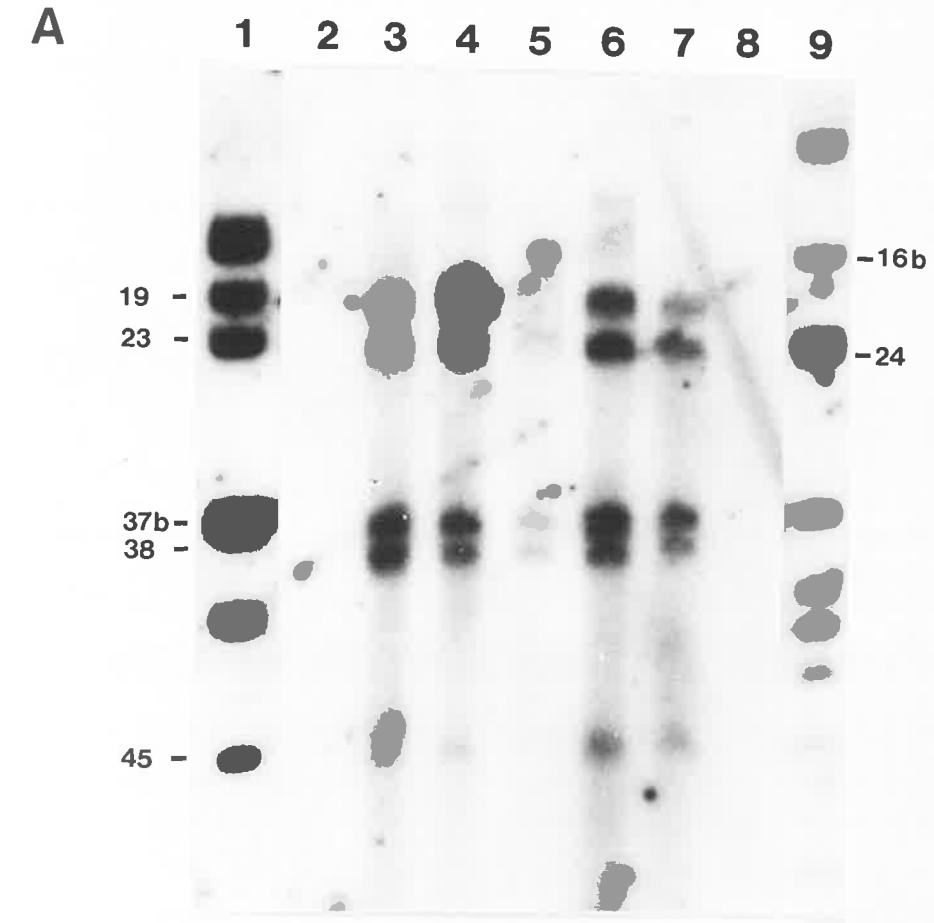
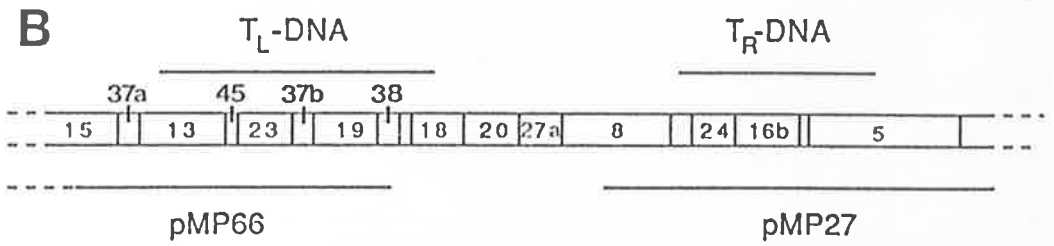

pUCD1186
absent
present
1855 + C58C1
vir -
1855 + LBA4404
vir +
tzs -
tzs +

Fig. 5-3. A. Southern hybridization analysis of independent root clones induced on flax cotyledon explants with A. rhizogenes strain 1855 in mixed inoculation with disarmed A. tumefaciens strain GV3850. Approximately 3-5 μg of plant DNA, digested by HindIII, was loaded in each lane and probed with a mixture of pMP66 and pMP27; 1-2 copy reconstructions of the probes digested with Hind III were used as markers. Lane 1, pMP66; Lane 2, control; Lanes 3-7, independent transformed root clones; Lane 8, untransformed root clone; Lane 9, pMP27. The numbers on the right correspond to restriction fragments in B. B. HindIII restriction map of the T-DNA regions of pRi1855, based on the map of Spanò et al. (1987).


A
1 2 3 4 5 6 7 8 9
19
23
37b
38
45
16b
24


B
T_L-DNA
T_R-DNA
37a
45
37b
38
15
13
23
19
18
20
27a
8
24
16b
5
pMP66
pMP27

There are at least three possible explanations for the root promoting effect of trans-zeatin. The first is that it stimulates cell division, so making plant cells more receptive to transformation by A.rhizogenes, i.e. a direct role in the transformation process. The second possibility is that more of the cells transformed by A. rhizogenes are triggered by the cell-division stimulus of trans-zeatin to actually divide and form roots. The third possibility is that the trans-zeatin secreted by A. tumefaciens, in addition to the auxin secreted by transformed plant cells, optimizes the conditions for induction of untransformed roots.

The last hypothesis is clearly the least likely since a prediction arising from it would be that the additional roots formed after mixed inoculation are unlikely to be transformed by A. rhizogenes. However, 5/6 or 85% of the root-derived callus clones obtained after mixed inoculation carried the T-DNA of pRi1855. Clearly, this is inconsistent with an hypothesis of additional root induction unrelated to transformation by A. rhizogenes.

Furthermore, addition of exogenous kinetin to cotyledon explants after inoculation with A. rhizogenes alone did not induce any more roots than without exogenous kinetin. Thus, exogenous cytokinin would appear to neither improve hormonal induction of untransformed roots nor to trigger cell division leading to root formation by transformed cells committed to root formation. For these reasons we favour the first hypothesis i.e. that trans-zeatin stimulates cell division making plant cells more receptive to transformation. This has the added attraction of being more consistent with the vir inducible nature of tzs, since it involves tzs directly in the vir inducible process of transformation rather than in events which occur later.

Clearly, these results may be used to support the notion that tzs, while not essential for transformation, does indeed promote transformation. In this example, the activity of the tzs gene in the disarmed strains of A. tumefaciens obviously supplements that of the weakly expressed tzs gene in A. rhizogenes strain 1855, leading to enhanced induction of transformed roots. The merit of using the tzs gene to promote transformation is the precision of its action. It acts at the right time, right

location and with the right concentration of zeatin, i.e. it applies a high concentration of zeatin to plant cells exposed to A. rhizogenes and at the time that the T-DNA of the Ri-plasmid is ready to transfer. This could never be achieved by just adding zeatin in the medium. A practical consequence of this observation for plant genetic engineering purposes is obvious. For situations where regeneration of plants is only possible or desirable via roots, transformation efficiency and consequently root induction efficiency can presumably be improved by including a tzs gene somewhere in the transformation system, providing that the tzs gene is exposed to vir induction.

The tzs gene joins a growing number of genetic components from various strains of A. tumefaciens which enhance transformation efficiency. A very similar example to that shown here has been the use of a cloned segment of the Vir region of the agropine Ti plasmid pTiBo542 to enhance the virulence of A. rhizogenes strain A4 (Pythould et al., 1987). This cannot have been due to the effects of tzs gene, however, since pTiBo542 has been shown not to carry tzs (Akiyoshi et al.,1987). Another example is the overdrive sequence adjacent to the right T-DNA border on octopine Ti plasmids (Peralta et al., 1986; Van Haaren et al., 1987).

CHAPTER 6 THE CONSTRUCTION OF SUBSTRATE REGULATED BINARY VECTORS

INTRODUCTION

A variety of vectors, including cointegrate vectors and binary vectors based on the disarmed Ti plasmid, have been constructed and used to introduce foreign DNA into plant cells (Pouwels et al., 1985; Klee et al., 1987; Bevan and Goldsbrough, 1987). Transformed cells grow into callus under selective pressure for a phenotype such as kanamycin resistance conferred by a gene carried on the introduced DNA and are then regenerated into transformed shoots. This strategy is widely used and has proved very successful. The disadvantages of this strategy are: (1) many species of plant rely on regeneration of shoots from roots (Tepfer, 1984) or regeneration of shoots from roots is much better than from callus (Chapter 4; Zhan et al., 1988); (2) callus induced after inoculation is usually chimeric, so a high proportion of untransformed plants will escape from selective pressure (Sacristan and Melchers, 1977; Van Slogteren et al., 1983; Horsch et al., 1985; Firoozabady et al., 1987; Chapter 4; Zhan et al.,1988); (3) genetic instability or somatic variation occurs during callus proliferation (Larkin and Scowcroft, 1981; 1983). A strategy based on the Ri plasmid overcomes these disadventages because roots induced by A. rhizogenes are: (1) almost all transformed (Petit et al., 1986 ; Chapter 4); (2) derived from single hairy root meristems and constitute cellular clones (Petit et al., 1986; Bercetche et al., 1987); (3) genetically stable during culture (Hänisch ten Cate and Ramulu, 1987; Hänisch ten Cate et al., 1987; 1988; Bercetche et al., 1987; Aird et al., 1988). However, overproduction of auxin by the auxin genes and the presence of the rol genes may interfere with shoot regeneration and cause gross morphological and physiological aberrations in the transformed plants.

In order to develop a new vector system preserving the advantages of vectors based on both the Ti and Ri plasmids but without their disadvantages, a set of new binary vectors has been constructed by introducing the iaaH gene from pTiA6 (Klee et

al., 1984; Sciaky and Thomashow, 1984) into the binary vector pDG12Sa (Vilaine and Casse-Delbart, 1987). The general strategy is as follows: Explants from many plants can be induced to root by auxin, so plant cells transformed by the iaaH gene, when cultured on a medium containing acetamide, which is a substrate for the iaaH gene, should produce auxin and initiate roots. Then these roots will be excised and transferred to differentiation medium without acetamide. Because the production of auxin by transformed cells is substrate regulated, efficient regeneration of shoots from these phenotypically normal roots should be expected; the transformed plants should also be normal both morphologically and physiologically. In addition to having iaaH with its own promoter the structural gene was also inserted behind the CaMV35S promoter (Odell et al., 1985) in both orientations. This might provide regulated synthesis of IAA over a wider concentration range.

MATERIALS AND METHODS

Plant material

The flax cultivar Bombay was used. Seeds were surface disinfected (see Chapter 2), then germinated and grown on hormone free Murashige and Skoog medim (MS)(1962) at 25 ^{0}C under a 16 h light (500 lux)/8h dark cycle.

Bacterial strains and plasmids

The bacterial strains and plasmids used are shown in Table 6-1.

Bacterial culture and matings

Agrobacterium strains were grown at 25 ^{0}C in Luria broth (LB) (Miller, 1972) without salt. E.coli strains were grown at 37 ^{0}C in LB. Antibiotics used were, for E.coli: ampicillin (40 µg/ml) and for Agrobacterium: rifampicin (100 µg/ml), kanamycin (100 µg/ml). An E.coli helper strain (RK2013) containing the mobilizing plasmid pRK2013 (Ditta et al.,1980) was used to mobilize plasmid constructs from E.coli to Agrobacterium. Triparental matings (Ditta et al., 1980) were carried out by

Table 6-1. Bacterial strains and plasmids.

Bacteria	Description	Source	Reference
Bacteria			
HB101	E.coli strain	D Jones	Boyer and Roulland-Dussoix,1969
1855	non-polar strain of Agrobacterium rhizogenes containing agropine Ri plasmid	J. De Ley	Spanò et al.,1982
LBA4404	non-pathogenic derivative of Ach 5 containing the disarmed octopine Ti plasmid pAL4404 which supplies Vir function to binary vector, rif^r, Sm^r	J. Ellis	Ooms et al.,1982
Plasmids			
p132-10	BamH1 fragment 8 of pTi A6Nc cloned in pBR322, Ap^r	D. Sciaky	Personal communication
pCaMVNEO	CaMV35S promoter with npt-ll gene and nos PolyA region cloned in pUCPiAN7, Ap^r	V. Walbot	Fromm et al.,1986
pDG12Sa	Binary vector with ColE1 ori and pArA4a ori	F. Vilaine	Vilaine and Casse-Delbart,1987
pRK2013	plasmid mobilizing strain	D. Jones	Ditta et al.,1980
pZX28	p132 -10 with Hind lll fragment Y deleted, Ap^r	This work	
pZX16	deleted BamH 1 fragment 8 of pZX28 inserted into Sal 1 site of pDG12Sa, Ap^r, Km^r	This work	
pZX15	Hind lll fragment from pZX28 containing gene iaaH replaced npt-ll gene in pCaMVNEO with the AUG initiation codon adjacent to CaMV35S promoter, Ap^r	This work	

Table 6-1. continued

pZX7	Hind lll fragment from pZX28 containing gene iaaH replaced npt-ll gene in pCaMVNEO, reverse orientation with pZX15, Ap^r	This work
pZX15-1	Xbal segment in pZX15 containing gene iaaH inserted into pDG12sa, Ap^r, Km^r	This work
pZX7-4	Xbal segment in pZX7 containing gene iaaH inserted into pDG12Sa, Ap^r, Km^r	This work

mixing 1ml of overnight liquid cultures of the donor, helper and recipent, and filtering the mixtures on to 0.45 μm pore size Millipore filters. The filters were incubated on non selective LB agar plates overnight at 28 ^{0}C. The bacteria were resuspended in 10 ml LB liquid medium, a ten fold dilution series prepared and 10 μl aliquots of appropriate dilutions plated on selective medium.

Recombinant DNA techniques

Large-scale preparation and cesium chloride density gradient purification of plasmids from E.coli were carried out according to Maniatis et al. (1982). Mini-preparation of plasmids from E.coli and Agrobacterium were performed as described by Maniatis et al. (1982) and Farrand et al. (1985) respectively.

Restriction endonuclease digestions were carried out according to the manufacturer's instructions and the conditions for partial digestions were worked out as described by Maniatis et al. (1982). Restriction fragments were separated by agarose gel electrophoresis according to Maniatis et al. (1982) and recovered using Gene Clean (BIO 101 INC. CALIFORNIA USA) according to the manufacturer's instructions. Treatment with calf intestinal phosphatase and ligation with T4 DNA ligase were carried out according to Maniatis et al.(1982). Competent cells of E.coli HB101 were prepared and transformed by protocol 3 of Hanahan (1985). To identify recombinant plasmids, transformants were analysed by restriction endonuclease digestion and agarose gel electrophoresis of plasmid mini-preparations, as described above.

Transformation and regeneration

Fresh bacterial cultures were centrifugated at 4000 x g for 10 min at 4 ^{0}C. Bacteria were resuspended in LB medium without salt and adjusted to 5 x 10^8 cfu/ml. Cotyledons from 7 day old seedlings were cut into halves and submerged in the bacterial suspension for one to two hours. After blotting dry, they were transferred to MS medium, and incubated at 20 ^{0}C in the dark for 2 days. They were then

transferred to MS medium containing 500 μg/ml cefotaxime supplemented with 0.5 mg/ml α - naphthaleneacetamide (Sigma) and incubated at 25 ^{0}C under a 16 h diffuse light/8h dark cycle. Roots, 0.5 - 1cm in length, which developed at the cut ends of cotyledons, were excised and placed on MS medium containing 0.02 mg/l naphthalene acetic acid (NAA), 1mg/l 6 - benzylaminopurine (6-BA) and 20 mg/l adenine and incubated at 25 ^{0}C under a 16 h light (500 lux)/8h dark cycle to allow shoot formation.

Plant DNA extraction and Southern blot analysis

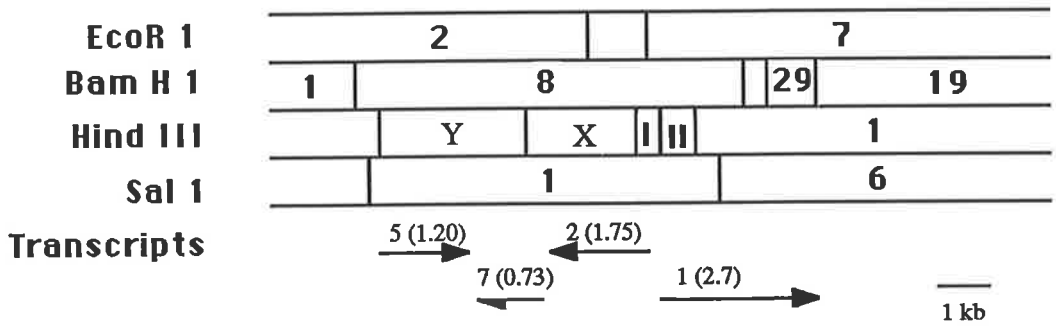
Plasmid pZX16 (see Table 6-1) was used as a probe of total DNA from calli induced at the cut ends of inoculated cotyledon explants or calli derived from roots developed from inoculated explants. Plasmids were prepared and purified by cesium chloride density gradient centrifugation according to Maniatis et al.(1982). Plant DNA extraction and Southern blot analysis were carried out according to Zhan et al. (1988; Chapter 4).

Results

Construction of vectors

The binary vector pDG12Sa was chosen as the basis of our vector because it contains both the Col E1 and pArA4a origins of replication (Vilaine and Casse-Delbart, 1987). So, it is able to replicate in both E.coli and Agrobacterium and is compatible with both Ti and Ri plasmids. The iaaH gene was isolated from plasmid p132-10 (Sciaky, personal communication) which comprises BamH1 fragment 8 of pTiA6NC cloned in pBR322 and contains two complete and one incomplete T-DNA genes beside iaaH (transcript 2 in Fig. 6-1). To remove the additional complete genes, p132-10 was partially digested with HindIII and the products separated by agarose gel electrophoresis. Fragments approximating the size predicted for p132-10 with a deletion of HindIII fragment Y were recovered from the gel using Gene Clean, self ligated and transformed into E.coli HB101. The construct designated pZX28, with a deletion of HindIII fragment Y, was identified among the transformants.

Fig. 6-1. Restriction endonuclease map of BamH1 fragment 8 in the T-DNA region of octopine plasmid pTiA6NC with the location and direction of 4 polyadenylated mRNA transcripts shown below (taken from Lichtenstein et al., 1984). Transcript 2 corresponds gene to iaaH.


EcoR 1
Bam H 1
Hind III
Sal 1
Transcripts
2
7
1
8
29
19
Y
X
I
II
1
1
6
5 (1.20)
2 (1.75)
7 (0.73)
1 (2.7)
1 kb

The reduced BamH1 fragment 8 was isolated from pZX28 by agarose gel electrophoresis of a BamH1 digest and recovered using Gene Clean. The binary vector, pDG12Sa, was digested with Sal1. The sticky ends of vector and insert were made compatible by partial fill in using dTTP and dCTP for the vector and dATP and dGTP for the insert. Both vector and insert were purified using Gene Clean, ligated and introduced by transformation into E.coli HB101. The construct pZX16 was identified among the transformants (Fig. 6-2).

To put the iaaH gene under control of the CaMV35S promoter, the plasmid pCaMVNEO (Fromm et al., 1986), which contains a CaMV35S promoter, an npt II coding region and a nos polyadenylation region, was digested with BamH1 to remove the 1kb npt II coding region. The vector, minus the npt II coding region, was separated by agarose gel electrophoesis, recovered using Gene Clean and ligated to a 1 : 4 mixture of phosphorylated BamH1-Sma1 and non-phosphorylated HindIII-Sma1 adaptors to create HindIII sticky ends, and recovered again using Gene Clean. The 2kb HindIII fragment X, containing the iaaH coding region and 51 nucleotides 5' to the coding region (3 nucleotides 5' to the TATA box) and about 500 nucleotides 3' to the coding region, was isolated from pZX28 by agarose gel electrophoresis of a HindIII digest and recovered using Gene Clean. The purified vector was ligated to fragment X and transformed into E.coli HB101. Constructs with both orientiations of the iaaH coding region relative to the CaMV promoter of the vector were identified among the transformants by agarose gel electrophoresis of EcoR1 digests. One construct with the iaaH initiation codon adjacent to the CaMV35S promoter was designated pZX15 and another with the reverse orientiation was designated pZX7. Both constructs were digested with Xbal, and fragments from each containing the CaMV 35S promoter, iaaH coding region and nos polyadenylation region were separated by agarose gel electrophoresis and recovered using Gene Clean. These fragments were each ligated into Xbal digested, phosphorylated and Gene Clean purified pDG12Sa and used to transform E.coli HB101. Constructs designated pZX15-1 and pZX7-4 were identified among the transformants (Fig. 6-3).

Fig. 6-2. Construction of pZX28 and pZX16. E, EcoR1; B, BamH1; X, Xbal; Sl, Sal1; P, Pst1; S, Sph1; H, Hindlll; Bl and Br represent the left and the right border sequences of T-DNA, respectively. pZX16 contains gene iaaH with its own promoter.

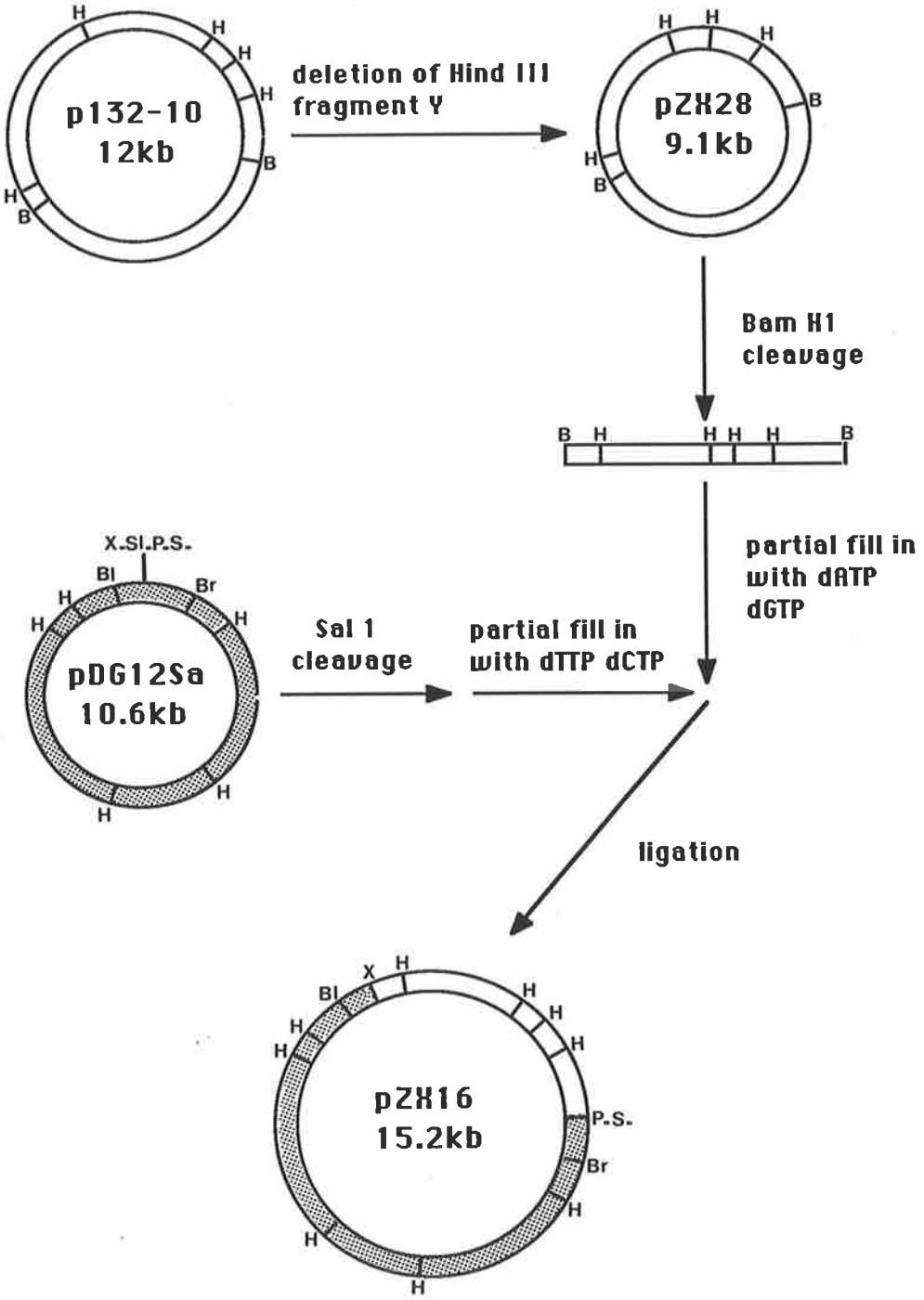
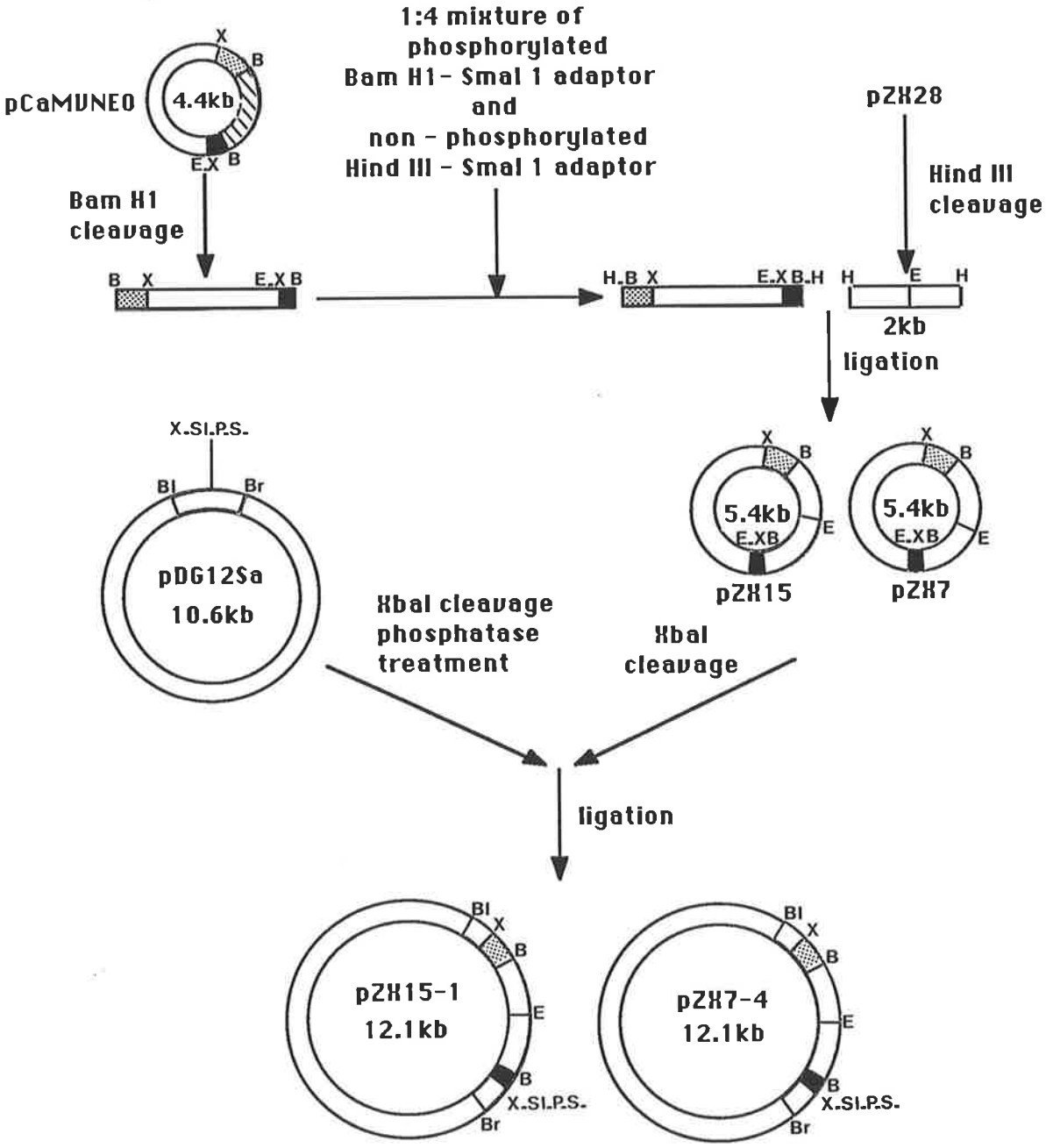

p132-10
12kb
deletion of Hind III
fragment Y
pZH28
9.1kb
Bam H1
cleavage
partial fill in
with dATP
dGTP
pDG12Sa
10.6kb
Sal 1
cleavage
partial fill in
with dTTP dCTP
ligation
pZH16
15.2kb

Fig. 6-3. Construction of pZX15-1 and pZX7-4. Stippled, cauliflower mosaic virus 35S promoter; hatched, neomycin phosphotransferase ll; black, nopaline synthase polyadenylation region; E, EcoR1; B, BamH1; X, Xbal; Sl, Sal1; P,Pst1; S,Sph1; H, Hindlll; Bl and Br represent the left and right border sequences of T-DNA, respectively.


pCaMVNEO
4.4kb
X
B
E.X
B
Bam H1
cleavage
1:4 mixture of
phosphorylated
Bam H1 - Sma I adaptor
and
non - phosphorylated
Hind III - Sma I adaptor
pZH28
Hind III
cleavage
B X
E.X B
H.B X
E.X B.H
H
E
H
2kb
ligation
X.SI.P.S.
Bl
Br
pDG12Sa
10.6kb
5.4kb
E.XB
pZH15
5.4kb
E.XB
pZH7
Xbal cleavage
phosphatase
treatment
Xbal
cleavage
ligation
pZH15-1
12.1kb
pZH7-4
12.1kb

The new binary vectors pZX16, pZX15-1 and pZX7-4 were transferred, by triparental mating using the mobilizing plasmid pRK2013, from E.coli to Agrobacterium strain LBA4404, it contains the disarmed octopine Ti plasmid pAL4404 which supplies Vir functions.

Transformation of flax cotyledon explants

Fifteen to 20 days after inoculation, explants inoculated with strains LBA4404 (pAL4404 ; pZX16) and LBA4404 (pAL4404 ; pZX15-1) developed callus and a lot of roots on medium containing 0.5µg/ml α-naphthaleneacetamide. No callus and few roots developed on the control explants, viz those inoculated with LBA4404 (pAL4404 ; pZX7-4) (with the iaaH gene in reverse orientation to the CaMV 35S promoter); or with LBA4404 (pAL4404) (no iaaH gene) on medium containing α-naphthaleneacetamide; or with LBA4404 (pAl4404 ; pZX16) (Fig. 6-4) when α-naphthaleneacetamide was omitted from the medium. The positive treatments induced more root production on explants than on those inoculated with A. rhizogenes. However, the most prolific root induction resulted when non-inoculated explants were cultured on medium containing 0.5µg/ml NAA (Fig. 6-4). Also, no callus and few roots developed on the explants inoculated with LBA4404 (pAL4404 ; pZX15-1) on medium without α-naphthaleneacetamide (data not shown). Differences between explants inoculated with strain LBA4404 (pAL4404 ; pZX16) and LBA4404 (pAL4404 ; pZX15-1) were not obvious. Southern analysis showed that callus developed from the cut ends of cotyledons inoculated with strains containing iaaH were transformed (Fig. 6-5). However, none of the analysed 20 independently induced roots from cotyledon explants inoculated with strain LBA4404 (pAL4404 ; pZX15-1) was transformed.

Discussion

Although transformed callus has been successfully induced using new vectors and the induction was substrate regulated, the attempt to induce transformed roots by

Fig. 6-4. Effects of gene iaaH on root induction from flax cotyledon explants. A. control cotyledon explants cultured on MS medium with 0.5 μg/ml NAA; B. control cotyledon explants cultured on medium with 500 μg/ml cefotaxime; C. control cotyledon explants cultured on MSCN medium; D, cotyledon explants inoculated with Agrobacterium strain LBA4404 (pAL4404 ; pZX16) and cultured on MSCN medium; E, cotyledon explants inoculated with Agrobacterium strain LBA4404 (pAL4404 ; pZX15-1) and cultured on MSCN medium; F, cotyledon explants inoculated with Agrobacterium strain LBA4404 (pAL4404 ; pZX7-4); G, cotyledon explants inoculated with Agrobacterium strain 1855 and cultured on MS medium with 500 μg/ml cefotaxime; H, cotyledon explants inoculated with Agrobacterium strain LBA4404 (pAL4404) and cultured on MSCN medium; I, cotyledon explants inoculated with Agrobacterium strain LBA4404 (pAL4404 ; pZX16) and cultured on MS medium with 500 μg/ml cefotaxime; MSCN, MS medium supplemented with 500 μg/ml cefotaxime and 0.5 μg/ml a-naphathleneacetamide.

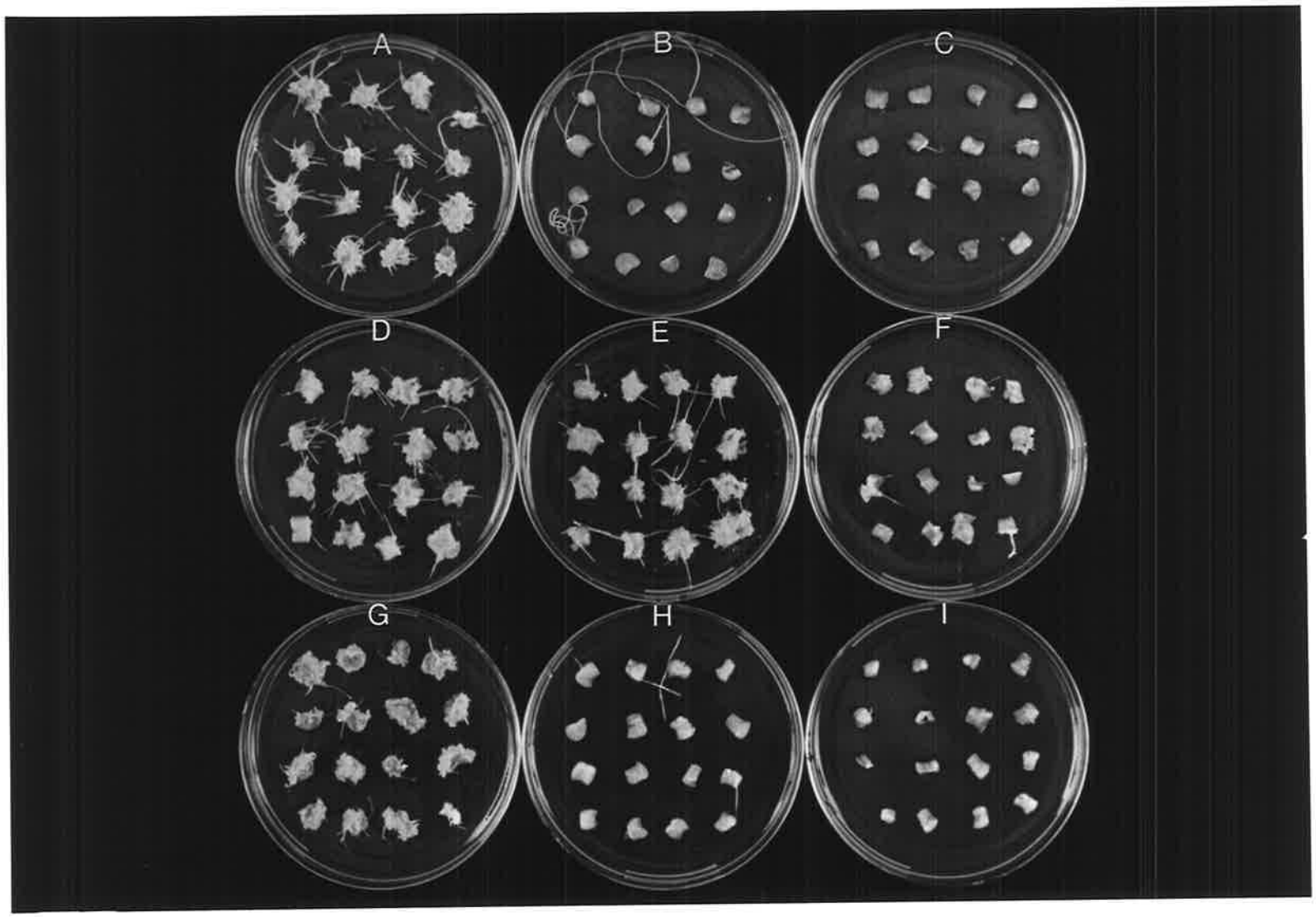
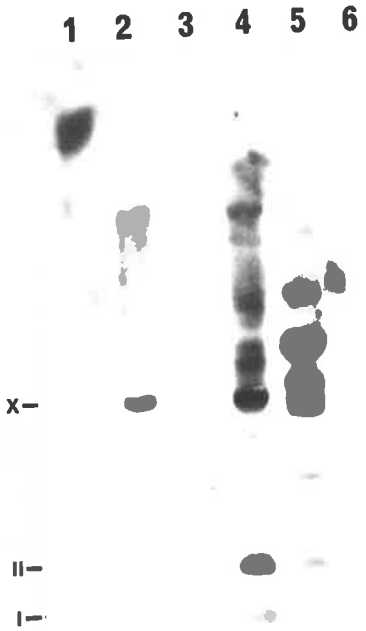
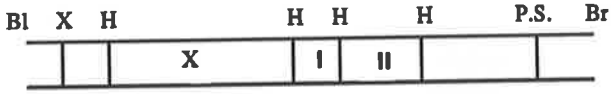

A
B
C
D
E
F
G
H
I

Fig. 6-5. A. Southern blot analysis of callus and roots induced from cut ends of flax cotyledon inoculated with Agrobacterium strains LBA4404 (pAL4404 ; pZX16) and LBA4404 (pAL4404 ; pZX15-1). Approximately 3-5 μg of plant DNA were loaded in each lane and probed with pZX16. DNA of lanes 1-2 were digested with BamH1, and DNA of lanes 3-6 were digested with Hindlll. Lane 1, root induced by LBA4404 (pAL4404; pZX15-1); lane 2, callus induced by LBA4404 (pAL4404; pZX15-1); lane 3, root induced by LBA (pAL4404; pZX16); lane 4, callus induced by LBA4404 (pAL4404; pZX16); lane 5, 1-2 copy reconstruction using Hindlll digested prob DNA; lane 6, untransformed Bombay callus. The symbols on the right correspond to restriction fragments in B and C. B. Hindlll restriction map of the T - DNA region of pZX16. C. BamH1 restriction map of the T - DNA region of pZX15-1. E, EcoR1; B, BamH1; X, Xbal; Sl, Sal1; P, Pst1, S,Sph1; H, Hindlll ; (H), lost Hindlll site during vector construction. Bl and Br represent the left and the right border sequences respectively.

A

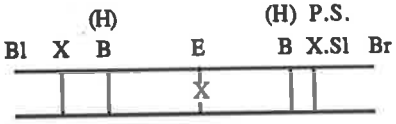


B



T - DNA region of pZX16

C



T - DNA region of pZX15-1

inoculation with these vectors from infected cotyledon explants failed. Roots developed directly from infected explants were not transformed. It seems that some untransformed cells form roots much more easily than do transformed cells. These cells could be: (1) already committed to form roots before inoculation, so they form roots easily under the stimulation of auxin excreted from transformed cells (Bercetche et al. 1987); (2) located close to veins, so that they were exposed to a high concentration of auxin which was excreted by transformed cells and transported polarly via veins, and induced to grow into roots.

The experiment showed that the iaaH gene could be used as a useful selection marker in transformation. On medium containing acetamide, cells transformed by the iaaH gene will produce auxin to support growth and proliferate rapidly while non-transformed cell cannot get enough auxin to support their growth. As a result, transformed callus developed from the inoculated explants as shown in Fig. 6-5.

Instead of inducing roots directly from inoculated explants with Agrobacterium containing the new vectors, a possible alternative would be to induce transformed roots from transformed callus by culturing the callus on medium with an appropriate level of acetamide. Transformed shoots could then be regenerated from these roots. However, as the procedure is via callus proliferation, somatic variation may occur. This could be a serious disadvantage to the ultimate aim of genetic engineering; for example, to transfer a gene for disease resistence to flax or to clone a gene for disease resistence from flax, since the somatic variation may change the interaction between flax and its pathogens.

Based on the understanding of the function of rolB and rolC (Schmülling et al., 1988), a binary vector(s) may be constructed by using rolB and rolC in which rolB and rolC are put under regulated control and inserted between T-borders. During the induction process, rolB and rolC should be switched on. Cells transformed by rolB and rolC will be very sensitive to auxin and will readily initiate roots. Then, rol genes could be switched off and transformed roots excised and transferred to differentiation medium for shoot regeneration. Since rol genes would not be active after switching

off, they would not interfere with regeneration and the morphology of the transformed shoots would be normal. Such vector(s) could be used in combination with pZX16 or pZX15-1 for further positive control of transformed root initiation.

CHAPTER 7 GENERAL DISCUSSION

As pointed out in Chapter 1, this project was part of a wider study whose aim was to clone a gene for resistance. Although disease resistance genes play an important role in crop production, the molecular basis is not known for any system and no product of a resistance gene has been isolated. Rather than make yet another attempt to isolate a resistance gene product , it may be better to clone a resistance gene and then investigate its products. This might be achieved by a transformation technique using a "shotgun" strategy (Jones et al., 1985; Simoens et al., 1986; Klee et al., 1987b; Prosen and Simpson et al., 1987) which has proved successful in cloning genes from bacteria and yeast. Flax was chosen because of the well characterized genetic control of the host - parasite interaction elucidated by Flor (1956). It has other properties that make it well suited for use in such an approach. It has a relatively small genome size (haploid genome size is 7 x 10^8 bp), so the number of clones required to encompass the genome should not be too large. As it is a dicotyledon, it can be transformed by Agrobacterium. The strategy proposed by Jones et al.(1985) is summaried as follows: First,the genome of a rust - resistant flax cultivar carrying four resistance genes that are not closely linked should be cloned into E. coli using a disarmed binary cosmid vector. Then the genomic library would be transferred in bulk from E.coli to Agrobacterium and from there in bulk to tissue explants of a rust-susceptible flax cultivar. The inoculated explants would be regenerated and the transformed regenerants inoculated with a strain of rust carrying the four corresponding avirulence genes. A plant transformed with a clone of a resistance gene would be identified by a hypersensitive response to the rust and the clone would be recovered, using the vector as a tag. To be 95% certain of recovering at least one clone of a unique gene sequence, assuming average cosmid insert size of 34kb and gene size of 1kb, 15887 clones are required or 21844 clones are required if the gene size is 10kb (Jone et al., 1985). The numbers of clones required might be even more because of instability of clones during transfer (Simoens et al, 1986), but on the other

hand, it could be reduced by multiple transformation of plant cells. If more than one clone could be transferred to one plant cell, then the number of plant cells which have to be transformed decreases by this factor (Depicker et al., 1985; Petit et al., 1986; Prosen and Simpson, 1987). No doubt, this is a large number of clones and would require a major effort, but might be just within the bounds of feasibility if an efficient transformation, regeneration, transplant and screen system were available.

In Chapter 2, it was clearly established that flax could be readily regenerated from various explants, confirming and extending the work that had already done by others (Link and Eggers, 1946; Gamborg and Shyluk, 1976; Rybczynski, 1975; Mathews and Narayanaswamy, 1976 and Barakat and Cocking, 1983). Buds could be regenerated efficiently from hypocotyl explants of all 10 cultivars that were tested. On average about 50 buds were regenerated from a 1 cm length of hypocotyl segment. Buds also could be regenerated from cotyledon explants, root explants and callus developed from protoplasts but with less efficiency. Linum marginale, a native Australian species, was also regenerated from hypocotyls, cotyledons, leaves and from protoplasts (Chapter 3). In Chapter 4 it was attempted to combine the efficient regeneration of flax from hypocotyls and from cotyledons with transformation by A. tumefaciens containing genes for opine synthesis and for resistance to kanamycin. Initial results were promising; buds were regenerated on medium containing kanamycin and nopaline analysis was positive. However, Southern analysis gave negative results. No transformant was detected from inoculated hypocotyls possibly because buds developed from hypocotyls are initiated from epidermal cells and the cutin layer outside the epidermal cells may have prevented the bacteria having access to these cells. Also, no transformed shoot from callus on inoculated hypocotyls or cotyledons was obtained, presumably because the regeneration rate of transformed callus was too low in the cultivars that were used. Such transfornation and regeneration has been reported by Basiran et al. (1987) and Jordan and McHughen (1988). Their successes were probably due to the flax cultivars that they used.

However, they presented no Southern analysis data and there must still be some doubt about their results.

Successful transformation and regeneration of flax was achieved using A. rhizogenes as described in Chapter 4. The results showed that transformation by A. rhizogenes is an effective alternative to transformation by a disarmed strain of A. tumefaciens for the genetic engineering of plants. However, the regeneration efficiency was low due, in part at least, to the presence of T-DNA of the Ri plasmid that had integreted into the genome of plant cells (Chapter 4) and would have to be greatly increased before the cloning of a resistence gene could be attempted. Also, the regenerated plants were physiologically abnormal and there is no guarantee that they would react to rust infection with the same specificity as the parent.

Flax cotyledon explants could be infected with a strain of A. rhizogenes containing a wild type Ri plasmid and a disarmed vector with foreign DNA between T - DNA borders, or infected with a mixture of both a wild type A. rhizogenes strain and a strain containing a disarmed vector. A high proportion of roots induced in this way should be multiple transformed by T-DNA of Ri plasmid and foreign genes from the binary vector (Depicker et al., 1985; Petit et al., 1986). Seeds from transformed plants would give rise to some plants containing only T-DNA from the disarmed vector (Petit et al., 1986). They would be phenotypically normal. This process would be more likely because of the observations of Tepfer (1984, 1987). He pointed out that plants of two different phenotypes could be regenerated from roots transformed by A. rhizogenes: T' phenotype and T phenotype. Plants of T' type have very wrinkled leaves and are quite stunted while plants of T type have moderately wrinkled leaves and are somewhat shorter than normal plants. T plants can be easily obtained from T' plants because lateral branches produced on T' plants will often revert to the T type. Seeds can be obtained from plants of T type without any difficulty. The results in chapter 3 also showed that flax of two different phenotypes had been regenerated and the recovery of 'normal' phenotype from abnormal phenotype was observed. These results support Tepfer's observation. It is not clear why plants transformed by A.

rhizogenes have two different phenotypes and why T' type can spontaneously change to T type. The most likely explanation is that the rol genes, which are believed to cause the abnormal phenotype in transformed plants, are methylated during this period, so their expression is repressed (Hepburn et al., 1985).

Another approach was adopted in Chapter 6. It was attempted to place transformation of flax under regulatory control by means of the substrate. Vectors were constructed with the iaaH gene between T-DNA border seqences. This gene synthesized IAA only when acetamide was added to the substrate. The strategy was to remove the acetamide following transformation and, as a result, any transformed roots would be phenotypically normal. This would improve the efficiency of regeneration (see Chapter 4) of plants which would also be phenotypically normal. Unfortunately, only transformed callus was obtained. This gave rise to a general proliferation of roots but all those tested were not transformed. Presumbly the IAA synthesized by the transformed callus stimulated root proliferation

The strategy is probably worth pursuing further. For example, the rol genes, particularly rolB and rolC, could be put under regulational control and be used in co-operation with vector pZX16 or pZX15-1 both of which contain the iaaH gene and which were constructed during the course of this work (Chapter 6). During the induction process, rol genes should be switched on. Cells transformed by rol genes would be very sensitive to auxin produced by gene iaaH transferred to the same cells or to cells close by and would readily initiate roots. Then rol genes could be switched off and transformed shoots regenerated from these roots should be morphologically and physiologically normal. A hopeful strategy to control the expression of rol genes is to put them under the control of a promoter from plant defense genes which react to changing environmental conditions (Kuhlemeier et al., 1987; Lamb et al., 1989). Plant defense genes are inducible by pathogen attack or by mechanical wounding to produce enzymes involved in the biosynthesis of lignin, flavonoids, phytoalexins and protease inhibitors (Kuhlemeier et al., 1987; Lamb et al., 1989). So, rol genes under the control of one the promoters of defense genes, such as one of the wounding

inducible genes (Keil et al., 1989), should be expressed after they are exposed to cells at the cut ends of the explants. Roots would be induced but plants regenerated from them should be normal because the rol genes should be silent after wound healing.

Even if the strategy were successful, it seems doubtful if the transformation and regeneration of flax would become efficient enough to satisfy the requirments of resistance gene cloning by a "shotgun" approach. The application of RFLP (Restriction Fragment Length Polymorphisms) linkage mapping (reviewed by Tanksley et al., 1989) might cause a substantial improvement of this strategy. Clones containing DNA segments with resistance genes could be identified or a small population of clones including clones with resistance genes could be selected. This will greatly reduce the number of gene clones which need to be handled. Unfortunately, no RFLP maps of flax are available.

Another possibility would be to use a DNA gun. As indicated in Chapter 2, flax can be regenerated very efficiently from hypocotyl explants. The cells which give rise to the buds are located in the epidermal layer (Link and Eggers, 1946). It might be possible to transform these cells by bombardment with DNA. It is an approach that is certainly worth trying.

The work presented in Chapter 5 arose from an observation following inoculation of cotyledon explants with a mixture of two strains. A disarmed A. tumefaciens strain GV3850 clearly promoted root initiation induced by a wild type A. rhizogenes strain 1855. By a process of elimination, this was shown to be due to the presence of gene tzs in strain GV3850. This gene is under the regulatory control of the Vir region and synthesizes trans-zeatin only when vir genes are also present. It is another example of how to improve the efficiency of plant transformation and woud be helpful to this project.

Plant transformation is only one of the problems involved in the genetic engineering of plants. Efficient regeneration of transformed cells is perhaps a bigger problem and is a significant bottleneck in Agrobactreium-mediated gene transfer. There are still many unanswered questions in plant tissue culture. What are the

intrinsic differences between plants, such as tobacco which is so easily regenerated into shoots while many other species of plant are almost impossible to regenerate in vitro? What genes, besides hormone synthesis genes, control the shoot regeneration (or embryogenesis) processes? What is the nature of the commitment status of cells to form specific structures? A great inspiration from the study of A. rhizogenes and rol genes of Ri plasmid is that genes do exist which are not involved in the synthesis of plant hormones but seem to control cell differentiation. It is possible that there are still some unknown genes, similar to rol genes, which direct cells to form shoots (or embryoids) in vitro. Their discovery would greatly improve the precision and potential of plant tissue culture and gene transfer.

Finally, even when the transformation and regeneration problems are solved, there remains the problem of what genes to manipulate. Three types of genes are presently available for improving the agronomic characters of plants. They are: Genes for herbicides resistance (De Block et al., 1987; Della Cioppa et al., 1987); genes for insect resistance (Vaeck et al., 1987; Hilder et al., 1987) and genes for viral resistance (Abel et al.,1986: Harrison et al., 1987; Nelson et al., 1988). However, genes for most important agronomic characters; for example, yield, quality, stress tolerance, pathogen resistance and photosynthetic efficiency, are still not available.

The emphasis of the present work was to develop a procedure which would enable resistance genes to be located and cloned. Although not entirely successful, the search for such useful genes must continue. There is little point in having a sophisticated technology for the genetic engineering of plants, if there are very few agronomically useful genes available.

APPENDIX A: CULTURE MEDIA

LB medium (Miller, 1972)

Bacto - tryptone	10.0g
Bacto - yeast extract	5.0g
NaCl	10.0g
Agar	15.0g
distilled water	to 1 litre
PH	7.5

MS medium (Murashige and Skoog, 1962)

Macronutrients

NH_4NO_3	1650mg
KNO_3	1900mg
$CaCl_2 \cdot 2H_2O$	440mg
$MgSO_4 \cdot H2O$	370mg
KH_2PO_4	170mg

Micronutrients

KI	0.830mg
H_3BO_3	6.200mg
$MnSO_4 \cdot 4H_2O$	22.300mg
$ZnSO_4 \cdot 7H_2O$	8.600mg
$Na_2MoO_4 \cdot 2H_2O$	0.250mg
$CuSO_4 \cdot 5H_2O$	0.025mg
$CoCl_2 \cdot 6H_2O$	0.025mg
EDTA - Ferric Salt	43.000mg

Organic compounds

Inosotol	100.000mg
Nicotinic acid	0.500mg
Pyridoxine·HCl	0.500mg
Thiamine·HCl	0.100mg
Sucrose	30000.000mg
Agar	7000.000mg
distilled water	to 1 litre
PH	5.8

MSP - 1 Medium (Barakat and Cocking, 1983)

Macronutrients

NH_4NO_3	1650mg
KNO_3	1900mg
$CaCl_2 \cdot 2H_2O$	440mg
$MgSO_4 \cdot H2O$	370mg
KH_2PO_4	170mg

Micronutrients

KI	0.830mg

H_3BO_3	6.200mg
$MnSO_4 \cdot 4H_2O$	22.300mg
$ZnSO_4 \cdot 7H_2O$	8.600mg
$Na_2MoO_4 \cdot 2H_2O$	0.250mg
$CuSO_4 \cdot 5H_2O$	0.025mg
$CoCl_2 \cdot 6H_2O$	0.025mg
EDTA - Ferric Salt	43.000mg

Organic compounds

Inosotol	100.000mg
Nicotinic acid	0.500mg
Pyridoxine·HCl	0.500mg
Thiamine·HCl	0.100mg
Mannitol	90000.000mg
NAA	2.000mg
6 - BA	0.500mg
distilled water	to 1 litre
PH	5.8

MB5P medium (Barakat and Cocking, 1983))

Macronutrients

$(NH_4)_2SO_4$	134mg
KNO_3	2500mg
$CaCl_2 \cdot 2H_2O$	588mg
$CaH_4(PO_4)_2$	252mg
$MgSO_4 \cdot H2O$	250mg
KH_2PO_4	136mg
$NaH_2PO_4 \cdot H2O$	150mg

Micronutrients

KI	0.750mg
H_3BO_3	3.000mg
$MnSO_4 \cdot H_2O$	10.000mg
$ZnSO_4 \cdot 7H_2O$	2.000mg
$Na_2MoO_4 \cdot 2H_2O$	0.250mg
$CuSO_4 \cdot 5H_2O$	0.025mg
$CoCl_2 \cdot 6H_2O$	0.025mg
EDTA - Ferric Salt	43.000mg

Organic compounds

Inosotol	100.000mg
Nicotinic acid	1.000mg
Pyridoxine·HCl	1.000mg
Thiamine·HCl	10.000mg
L - glutamine	730.000mg
N - Z - amine	250.000mg
Sucrose	20000.000mg
Glucose	63000.000mg
NAA	0.200mg
2, 4 - D	0.100mg
zeatin	0.100mg
distilled water	to 1 litre
PH	5.8

APPENDIX B: PUBLICATIONS

Zhan X, Jones D A and Kerr A: Regeneration of flax plants transformed by Agrobacterium rhizogenes. Plant Molecular Biology . 11:551-559 (1988).

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