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Generation of Transgenic Cassava Having Reduced Cyanide Toxicity

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### 3. Executive Summary:

Our objective has been to generate transgenic cassava plants having reduced cyanide toxicity for human consumption. Our strategy was to isolate and over-express the gene encoding linamarase, the enzyme which converts linamarin into acetone cyanohydrin. The laboratory has achieved most of its objectives as well as expanded on those originally proposed. We have generated a cassava cDNA library and have isolated cDNA clones for linamarase. Southern blot analyses indicate that there is only one copy of the gene, despite the fact that there are three linamarase isozymes. In the last year we have also purified and characterized cassava hydroxynitrile lyase (HNL). HNL converts acetone cyanohydrin, one of the products of linamarase activity, into cyanide and acetone. A cDNA clone of HNL is currently being isolated. We have determined that acetone cyanohydrin is stable at low pH and temperatures, conditions which exist during fermentation of cassava. As recently determined by Dr. Hans Rosling's group, acetone cyanohydrin is an important contributor to the cyanogenic potential of processed cassava. It is, therefore, apparent that HNL activity in cassava is also a determinant of the cyanogenic potential of processed cassava. In addition, we have characterized the physiological basis for varietal differences in the cyanogenic potential (CNp) of cassava. We have shown that high CNp varieties synthesize and transport greater amounts of linamarin from young leaves than do low cyanogenic varieties. Older leaves apparently do not synthesize linamarin. In addition, we have re-examined the ability of roots to synthesize linamarin and have determined that low CNp, unswollen roots are capable of synthesizing linamarin at rates equivalent to young, high CNp leaves. With respect to our efforts to transform cassava, we have characterized various gene promoters as well as characterized transient expression assay systems and regenerated cassava from somatic embryos. In collaboration with Dr. Stan Gelvin and with financial support of the Rockefeller Foundation we have identified *Agrobacterium* Ti plasmids with enhanced transformation efficiencies. Our efforts in cassava transformation have been greatly facilitated by our association with Dr. William Roca at the Center for International Tropical Agriculture (CIAT) in Cali, Colombia. Dr. Roca has identified cassava varieties which can be efficiently regenerated. Using Dr. Roca's protocols we routinely can regenerate intact plants from somatic embryos derived from apical meristematic tissues. Once we have constructed more efficient transformation vectors these will be sent to CIAT for the genetic manipulation of cassava.

#### 4. Research Objectives:

Although the caloric content of cassava (*Manihot esculenta*, Crantz) food products is high, the presence of cyanogenic glycosides is potentially harmful to the consumer. Recently, it was demonstrated that there was a correlation between the cyanogen content of poorly processed cassava and the occurrence of a CN induced neurological disorder called Konzo (Tylleskar et al., 1992). Cassava contains two cyanogenic glycosides, linamarin and lotaustralin. Linamarin accounts for 95% of the total cyanogen content and is present in all tissues (Balagopalan et al., 1988). The site of linamarin synthesis has generally been assumed to be only the leaves and petioles (Koch et al., 1992; Makame et al., 1987). This assumption implied that the linamarin content in roots was determined by the flux of linamarin from the top of the plant to the root. However, this interpretation was difficult to resolve with the fact that linamarase, the enzyme which hydrolyzes linamarin is localized in the cell wall (Mkpong et al., 1990). The product of linamarase activity is acetone cyanohydrin. Acetone cyanohydrin spontaneously decomposes to HCN and acetone at pHs greater than 4.0 or temperatures greater than 30 °C. In addition, acetone cyanohydrin can also be enzymatically converted to HCN through the activity of hydroxynitrile lyase (HNL). Therefore, the apoplastic transport of linamarin would be expected to be cyanogenic. In order to understand the physiological basis for varietal differences in CNp it was necessary to determine the mode of transport, if any, of cyanogenic glycosides from leaves to roots.

Cyanogenesis or hydrolysis of linamarin is generally thought to provide protection against insect and/or herbivore attack (Arias and Belloti, 1984). Since cyanide is not released from undamaged tissues, it is believed that the cyanogenic glycosides and their glucosidases are differentially compartmentalized in the cell. Since linamarase is apoplastic, linamarin transport must either be symplastic or occur as a chemically modified, non-hydrolyzable form of linamarin. Recently, Selmar (1988) has demonstrated the presence of linustatin, a glucoside of linamarin, in *Hevea* seedlings. Linustatin is not a substrate for linamarase and therefore can move across the apoplast (which contains linamarase). It has been proposed that linustatin may be the form by which cyanogenic glycosides could be transported in cassava (Selmar, 1993).

In contrast to linamarase, the cellular localization of HNL has not been determined in cassava. Previous studies (Saunders and Conn, 1978) have indicated that dhurrin, the cyanogenic glycoside in *Sorghum*, is localized in the vacuoles. In cassava, acetone cyanohydrin, the immediate precursor to linamarin, is probably synthesized on the tonoplast membrane (Möller, personal communication) suggesting that linamarin may be stored in the vacuole. We have developed a method for isolation of vacuoles from cassava and have quantified the linamarin content of vacuoles.

As previously indicated, acetone cyanohydrin is present at potentially toxic levels in poorly processed cassava (Tylleskar, 1992). This observation was surprising since it was assumed that acetone cyanohydrin spontaneously or enzymatically decomposed to cyanide. In order to understand this discrepancy, we have characterized the stability of acetone cyanohydrin at various temperatures and pHs and characterized the biochemical properties and tissue specific localization of HNL.

Finally, it was our objective to reduce the CNp of cassava food products by over-expressing the cyanogenic enzymes in transgenic plants. Such plants would have reduced CNp with little or no changes in processing technology. This strategy is unique and remains to be tested in vivo but has been shown to be feasible by in vitro experiments. Presently, we are enhancing the transformation technology of cassava by using improved strains of *Agrobacterium* Ti plasmids. This work is supported by the Rockefeller Foundation.

In summary, our research objectives were:

- 1) Isolation, characterization and localization of linamarase and hydroxynitrile lyase and isolation of their corresponding genes.

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- 2) Characterization of cyanogenic glycoside synthesis and transport in low and high cyanogenic varieties of cassava
- 3) Development of a cassava regeneration and transformation system.
- 4) Introduction and over-expression of linamarase and hydroxynitrile lyase in cassava.

## 5. Methods and Results:

Three graduate students and a technician have worked on the. Two of the students have worked on the project for the last 30 months and the third joined the project in April, 1992. Ms. Diana Arias, formerly from CIAT, was supported as a graduate research assistant on the grant and the other two students, Ms. Jennifer McMahon and Ms. Wanda (Broyles) White, were supported as teaching assistants by the Department of Plant Biology. In addition, Ms. Evangeline Ricks, a technician from Liberia, was trained in molecular techniques and has recently enrolled in a selective training program for minorities entering medical school.

Diana Arias worked on the development a cassava transformation and regeneration system and on screening various gene promoter constructs to identify strong tissue specific gene promoters in cassava. Diana has been successful in regenerating plants from apical meristems and from somatic embryos. The successful development of regeneration protocols from somatic embryos is a significant result in meeting the objectives of our proposal since transformation of these tissues will result in the generation of plants which could over-express linamarase and HNL. Using B-glucuronidase and luciferase reporter genes, Diana has also been comparing various tissue specific gene promoters in transient expression assays using the particle gun transformation system. Significantly, she has shown that transient gene expression in cassava root tissues occurs at significantly lower frequency than in leaves. This is probably due to the presence of high levels of DNase activity, which we believe reduces the efficiency of transformation. These observations were published in *Plant Science* (see enclosed publication).

Ms. Jennifer McMahon characterized the dynamics of linamarin synthesis and transport in low and high CNp varieties of cassava. She has determined that linamarin is synthesized at 4-5 fold higher rates in young leaves of high cyanide varieties than in low CNp varieties. Interestingly, old leaves (>60 days following full expansion) of high and low cyanide varieties do not synthesize linamarin. These results indicate that temporal differences in cyanogenic glycoside synthesis may determine whether roots will have low or high cyanogen content. In addition, she has demonstrated that the form of cyanogenic glycoside which may be transported from the leaves to the roots is most likely linamarin and not its glucoside derivative, linustatin, as occurs in cassava's close relative, rubber tree. However, Jennifer also demonstrated that roots can synthesize linamarin at rates equivalent to those observed in leaves. This result has significant implications for the regulation of the cyanogenic potential of roots. As we have previously shown both low and high root CNp varieties have similar leaf (high) CNp. Jennifer has also demonstrated that linamarin can be synthesized by protoplasts and is sequestered in vacuoles (*Acta Horticulturae*, in press). Her results indicate that the regulation of cyanogen content in roots is most likely determined by biochemical processes occurring in both leaves and roots. In addition, she has isolated cDNA clones and a genomic clone of linamarase using polymerase chain reaction procedures. Our plans are to put the gene into Ti plasmid transformation vectors for generation of transgenic cassava which over-express this enzyme as originally proposed in our grant.

Recently, Tylleskar *et al.* reported in the journal *Lancet* (1992) that consumption of inadequately processed cassava can lead to high levels of thiocyanate (cyanide) in the blood. Toxic levels of cyanide in the blood can cause konzo, a paralytic disorder, or result in death. Significantly, they demonstrated that the cyanide poisoning associated with cassava consumption is due to the presence of two cyanogenic compounds in the

processed cassava: these are linamarin (50%) and acetone cyanohydrin (50%) (there was no free cyanide). The presence of acetone cyanohydrin at such high levels in processed cassava was unexpected. Acetone cyanohydrin is very unstable and can spontaneously decompose to cyanide and acetone, both of which are volatile. These results indicate that the levels of hydroxynitrile lyase (HNL) activity, the enzyme which converts acetone cyanohydrin to cyanide, may also be limiting for efficient removal of this compound. As a result, we initiated a project to isolate, characterize, clone and over-express this enzyme in cassava similar to our project with linamarase. Wanda White has purified and characterized HNL. She has demonstrated that it is present in apoplast extracts of leaves, i. e. it is most likely localized in the cell wall similar to linamarase. HNL is also very stable and is present in cassava leaf tissues at levels comparable to those of linamarase. These results suggest that processing or detoxification of cassava could be enhanced by elevation of both linamarase and HNL. Wanda has also determined the stability of acetone cyanohydrin in solution. Acetone cyanohydrin is stable only at low pH and temperatures: conditions which are found in fermenting cassava to be used for human consumption. Therefore, either elevated HNL levels or increased accessibility to the substrate should effect the removal of the cyanogens. Currently, she is isolating a cDNA clone for HNL.

The last person to mention whom has been assisting on this project is the technician, Ms. Evangeline Ricks, a political exile from Liberia. She has learned a number of protein chemistry and molecular biology techniques as well as tissue culture procedures for cassava. She works most closely with Diana Arias in plant regeneration and in gene isolation over the course of the project.

## **6. Impact, Relevance and Technology Transfer:**

Briefly, the most significant accomplishments of our work include: 1) the isolation, characterization and localization of HNL, 2) the isolation of cDNA clones for linamarase, 3) the characterization of the stability of acetone cyanohydrin in low pH media and its relevance to cassava processing procedures 4) the demonstration that roots can synthesize linamarin, 5) the development of cassava regeneration systems suitable for transformation and the identification of suitable Ti plasmid transformation vectors. Our studies and those of others have lead to the development of a set of recommendations (from the International Workshop on Cassava Safety held at IITA in Nigeria in March, 1994) for the application of recombinant DNA technology to the problem of cyanide toxicity in cassava. The Working Group on Cassava Safety recommends that a strategy based on tissue specific inhibition of linamarin synthesis combined with tissue specific over-expression of the cyanogenic enzymes, linamarase and HNL, is the most effective way to detoxify cassava using a recombinant DNA approach. These recommendations are identical in substance to those stated in our proposal, and are close to realization with the eventual introduction and over-expression of the genes encoding the cyanogenic enzymes in transgenic cassava.

In addition, the reports presented at the International Workshop on Cassava Safety established that there is ample evidence for acute and chronic disease associated with poorly processed or cyanogenic cassava, especially in Africa. As many as seven thousand cases of permanent paralysis, Konzo, associated with eating poorly processed cassava have been documented. In addition, goiter associated with thiocyanate interference of iodine metabolism; malnutrition, associated with protein requirements for cyanide detoxification, and tropical ataxic neuropathy are caused by eating poorly processed cassava. Cassava, however, is the only food security for most of sub-Saharan Africa. It grows in poor tropical soils, it is drought tolerant and insect resistant and can be left in the soil for long periods (3 years) of time without decay. Furthermore, the presence of cyanogens acts as a deterrent against theft. As a result, cassava accounts for 60-80% of the total caloric input for people living in sub-Saharan Africa. Due to its importance it is imperative that the problems of cyanide toxicity be addressed. In this context, a recombinant plant with reduced CNp is highly desirable and our progress towards that goal will

have an impact on the health of many persons in Africa. At present, we have achieved 70% of our objectives and are close to developing recombinant plants over-expressing the cyanogenic enzymes.

Another aspect of our program was the training of students from developing countries. Ms. Diana Arias, a native of Cali, Colombia, has been admitted to the Ph. D. program in Plant Biology. Ms. Arias has made good progress in her research and has published two papers after three years in the program. Diana's long term objectives are to return to Colombia or another South American country to an academic position in plant biotechnology. Our collaboration with Dr. Willie Roca at CIAT has also been fruitful and has included the exchange of plant materials and information on cassava regeneration and transformation. Currently, we are also collaborating on a research grant funded by the Rockefeller Foundation with Dr. Stan Gelvin to increase the efficiency of cassava transformation. Once this objective is realized we will be able to introduce new recombinant cassava varieties into developing countries for field tests of their CNp.

## 7) Project Activities/Output:

### Research Papers Presented at Scientific Meetings over the Entire Grant Period:

- 1) McMahon, J.M. and Sayre, R.T. (1992) Characterization of the synthesis and transport of cyanogenic glycosides in high and low cyanide cultivars of cassava. Annual Meeting of Am. Soc. Plant Physiol., Pittsburg, PA.
- 2) Arias-Garzon, D. I. and Sayre, R.T. (1992) Correlation between the frequency of transient gene expression and DNase activity in cassava tissues. Annual Meeting of Am. Soc. Plant Physiol., Pittsburg, PA.
- 3) McMahon, J. M. and Sayre, R. T. (1992) Differential biosynthesis and transport of linamarin in high and low cyanide cultivars of cassava. Cassava Biotechnology Meeting, Cartagena, Colombia.
- 4) White, W. L. and Sayre, R.T. (1992) Partial purification and characterization of hydroxynitrile lyase from cassava. Cassava Biotechnology Meeting, Cartagena, Colombia.
- 5) Arias, D. I. and Sayre, R.T. (1992) Correlation between the frequency of transient gene expression and DNase activity in cassava. Cassava Biotechnology Meeting, Cartagena, Colombia.
- 6) Sayre, R.T. (1993) Cyanogenesis in cassava. Seminar in the Dept. of Agronomy, Ohio State Univ.
- 7) White, Wanda and R.T. Sayre (1993) Partial purification of hydroxynitrile lyase from cassava and the spontaneous decomposition of its substrate acetone cyanohydrin. ASPP Annual Meeting, Minneapolis, MN
- 8) McMahon, J. and R.T. Sayre (1993) Linamarin synthesis in protoplasts of cassava (*Manihot esculenta*, Crantz) ASPP Annual Meeting, Minneapolis, MN.
- 9) Sayre, R.T. (1994) Regulation of cyanogenesis in cassava. Invited symposium speaker for International Workshop on Cassava Safety International Institute of Tropical Agriculture, Ibadan, Nigeria

### Publications over the Entire Grant Period:

- 1) McMahon, J.M. and Sayre, R.T. (1993) Differential biosynthesis of linamarin in low and high cyanide cultivars of cassava in *Proceedings of the First International Scientific Meeting of the Cassava Biotechnology Network*: Roca, W.M. and Thro, A.M. eds., pgs. 376-378

- 2) Arias-Garzon, D. I. and Sayre, R.T. (1993) Differential inhibition of transient gene expression in cassava root and leaf tissues. in: *Proceedings of the First International Scientific Meeting of the Cassava Biotechnology Network*; Roca, W.M. and Thro, A.M. eds., pgs. 239-243.
- 3) White, W.L.B. and Sayre, R.T. (1993) Partial purification and characterization of hydroxynitrile lyase from cassava. in: *Proceedings of the First International Scientific Meeting of the Cassava Biotechnology Network*; Roca, W.M. and Thro, A.M. eds., pgs. 379-383.
- 4) Arias, D. I. and Sayre, R.T. (1993) Tissue specific inhibition of transient gene expression in cassava (*Manihot esculenta* Crantz) tissues. (*Plant Science* 93:121-130).
- 5) White, W., McMahon, J., and Sayre, R.T. (1994) Regulation of cyanogenesis in cassava. Proceedings of the International Workshop on Cassava Safety. IITA, Ibadan, Nigeria (*Acta Horticulturae*, in press)

## 8 and 9. Project Productivity and Future Work:

As indicated earlier, we have accomplished approximately, 70% of our goals. We have isolated a linamarase cDNA clone, we have developed cassava regeneration technology and we have further characterized the regulation of CNp in low and high CNp varieties of cassava. The last barrier to completion of our initial objectives is the generation of a transgenic cassava over-expressing linamarase. We have, however, expanded the project with the discovery that HNL activity would also need to be enhanced to develop a low CNp cassava food product. We expect to have identified *Agrobacterium* Ti plasmid vectors which will permit efficient transformation of cassava within the next year. At that time we will be able to determine whether we can over-express linamarase and HNL in cassava and whether it will facilitate the removal of cyanogens from cassava.

## References

1. Arias, B. and Belloti, A.C., 1984. Aspectos ecologicos y de manejo de *Cyrtomenus bergi* Froeschner, chinche de la viruela en el cultivo de la yuca *Manihot esculenta* Crantz. CIAT Publication, Cali, Colombia.
2. Balagopalan, C., Padmaja, G., Nanda, S. and Morthy, S., 1988. Cassava in Food, Feed and Industry CRC Press, Boca Raton, Florida.
3. Koch, B., Nielson, V.S., Halkier, B.A., Olsen, C.E. and Moller, B.L., 1992. The biosynthesis of cyanogenic glycosides in seedlings of cassava (*Manihot esculenta* Crantz). Arch. Biochem. Biophys., 292: 141-150
4. Makame, M., Akoroda, M.O. and Hahn, S.K., 1987. Effects of reciprocal stem grafts on cyanide translocation in cassava. J. Agric. Sci. Camb., 109: 605-608
5. Mkpogon, O.E., Yan, H., Chism, G. and Sayre, R.T., 1990. Purification, characterization, and localization of linamarase in cassava. Plant Physiol., 93: 176-181.
6. Saunders, J.A. and Conn, E.E., 1978. Presence of the cyanogenic glycoside dhurrin in isolated vacuoles from *Sorghum*. Plant Physiol., 61: 154-157.

7. Selmar, D., 1993. Transport of cyanogenic glycosides: linustatin uptake by *Hevea* cotyledons. *Planta*, 191: 191-199.
8. Selmar, D., Lieberei, R. and Biehl, B., 1988. Mobilization and utilization of cyanogenic glycosides. *Plant Physiol.*, 86:711-716.
9. Tylleskar, T., Cooke, R.D., Banea, M., Poulter, N.H., Bikangi, N., and Rosling, H., 1992. Cassava cyanogens and konzo, an upper motoneuron disease found in Africa. *Lancet*, 339: 208-211.

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REGULATION OF CYANOGENESIS IN CASSAVA

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## Abstract

The presence of cyanogens in poorly processed cassava (*Manihot esculenta* Crantz) food products can cause neurological disorders such as konzo. Linamarin, the predominant cyanogenic glycoside in cassava, is present in all organs of the plant. The hydrolysis of linamarin by a specific -glucosidase, linamarase, results in the production of acetone cyanohydrin. Acetone cyanohydrin can decompose to HCN and acetone spontaneously or enzymatically by hydroxynitrile lyase. It had been assumed that linamarin synthesis was restricted to the leaves, and that linamarin present in roots was transported from the leaves. We have found that root tissues are capable of synthesizing linamarin at rates comparable to those in leaves. The presence of linamarase in the apoplast eliminates the apoplastic transport of linamarin, unless linamarin is modified (glycosylated) to form a non-hydrolyzable compound prior to transport. Labeling experiments using a radioactive precursor (valine) to linamarin indicate that linamarin is not glycosylated. Furthermore, we have demonstrated that linamarin is stored in the vacuole. Similar to linamarase, we show that hydroxynitrile lyase is localized in the apoplast. Hydroxynitrile lyase was purified to apparent homogeneity. Hydroxynitrile lyase has optimal activity at pH 5.0 and temperature of 35 °C. Similar to linamarase, hydroxynitrile lyase is quite stable. Our analyses of the stability of acetone cyanohydrin and activity of hydroxynitrile lyase suggest that cassava processing at pHs > 5.0 or in the presence of additional hydroxynitrile lyase would reduce the level of this cyanogen in food products.

Key Words: cyanide, linamarin, linamarase, acetone cyanohydrin, hydroxynitrile lyase

## I. Introduction

Cassava is cultivated throughout the tropics primarily as a source of starch (root) for human consumption. Although the caloric content of cassava (*Manihot esculenta*, Crantz) food products is high, the presence of cyanogenic glycosides can be hazardous to the consumer. In some regions of the world the presence of cyanogens in poorly processed cassava is associated with the occurrence of neurological disorders such as Konzo (Tylleskar et al., 1992). There are two different cyanogenic glycosides present in cassava, linamarin and lotaustralin. Linamarin accounts for 95% of the total cyanogen content and is present in all tissues (Balagopalan et al., 1988). The site of linamarin synthesis has generally been assumed to be the leaves and petioles implying that the linamarin content of roots is determined by its flux from the top of the plant (Koch et al., 1992; Makame et al., 1987). This assumption is difficult to resolve, however, with the observation that linamarase, the cyanogenic enzyme which hydrolyzes linamarin, is localized in the cell walls or apoplast of leaves (Mkpong et al., 1991). The immediate product of linamarase activity is acetone cyanohydrin, however, acetone cyanohydrin can spontaneously decompose to HCN and acetone at pHs greater than 4.0 or temperatures greater than 30 °C. Therefore, hydrolysis of linamarin can be cyanogenic. In addition, acetone cyanohydrin can be enzymatically converted to HCN through the activity of hydroxynitrile lyase (HNL).

In general, cyanogenesis only occurs in physically damaged tissues, indicating that the cyanogens (linamarin and acetone cyanohydrin) and cyanogenic enzymes (linamarase and HNL) are differentially compartmentalized in the cell. Since linamarase is apoplastic, linamarin is probably localized in the symplast. Alternatively, linamarin may be present in the apoplast in a chemically modified form, e.g. linustatin (the glucoside of linamarin) which can not be degraded by linamarase. Recently, Selmar (1988) demonstrated the presence of linustatin in the seedlings of the related genus, *Hevea*. As shown by Selmar et al. (1988), linustatin is transported from the seed to the growing shoot in *Hevea* seedlings. It has been proposed that linustatin may also be the transportable cyanogenic glycoside in cassava (Selmar, 1993). However, we have not detected linustatin in any extracts of cassava roots, leaves, or petioles. If linustatin is not present in cassava then it is probable that linamarin is not accessible to the apoplast.

It has been demonstrated that cyanogenic glycosides of some plants are stored in vacuoles. For example, dhurrin, the cyanogenic glycoside of *Sorghum*, was shown by Saunders and Conn (1978) to be localized in vacuoles. In cassava, acetone cyanohydrin, the immediate precursor to linamarin, is probably synthesized on the tonoplast membrane (Møller, personal communication) suggesting that linamarin is stored in the vacuole. Our analyses of the linamarin content of isolated cassava vacuoles indicate that the vacuole is the site of linamarin compartmentalization in cassava.

As previously mentioned, the presence of acetone cyanohydrin in processed cassava is surprising since it was assumed that acetone cyanohydrin would spontaneously or enzymatically decompose to cyanide (Tylleskar, 1992). In order to understand the factors which determine acetone cyanohydrin turnover, we have characterized the stability of acetone cyanohydrin at various temperatures and pHs as well as the biochemical properties and tissue specific localization of HNL. Our results indicate: 1) that acetone cyanohydrin is stable only at low pHs and temperatures (< 30 °C), 2) that HNL is also apoplastic, 3) that the apoplastic activity of HNL is greater than that of linamarase, and 4) that HNL is active at the low pHs (4.0) at which acetone cyanohydrin would otherwise be stable during fermentation.

## 2. Results and Discussion

### 2.1. Linamarin storage in the vacuole

Cassava protoplasts are between 0.14 and 0.2 mm in diameter and contain one large central vacuole which readily takes up neutral red. Upon detergent-induced release from the protoplasts the vacuoles increased in diameter from 0.22 - 0.56 mm equivalent to a 20-fold increase in volume. We determined the amount of linamarin present in vacuoles and protoplasts by measuring the amount of free cyanide released after extraction in boiling methanol and incubation with linamarase. As shown in Table 1, the quantity of HCN released from cassava vacuoles (0.16 mol HCN/10<sup>6</sup> vacuoles) was approximately 15% of that present in *Sorghum* vacuoles (1.13 mol HCN/10<sup>6</sup> vacuoles) (Saunders and Conn, 1978). Oddly, the amount of HCN released from cassava protoplasts was six fold (0.025 mol HCN/10<sup>6</sup> protoplasts) less than

that from protoplasts. The lower levels of cyanogens in protoplasts relative to vacuoles could be due to a detergent-induced fusion of vacuoles during their isolation or enrichment for vacuoles with a higher linamarin content. These results demonstrate, however, that vacuoles contain more than sufficient quantities of linamarin to account for the total present in individual cells.

## 2.2. Organ specificity of linamarin synthesis

In order to address the question of how linamarin might be transported from plant shoots and leaves to roots, we re-examined the ability of cassava roots to synthesize linamarin. We report that root tissues labeled with  $^{14}\text{C}$ -valine (0.5 mM) synthesize linamarin at rates ( $0.9 - 1.1 \times 10^{-3} \mu\text{mol/gfw/h}$ ) similar to those of leaves ( $0.45 - 0.984 \times 10^{-3} \mu\text{mol/gfw/h}$ ) from young plants (49 days after germination) ( See Table 2). It is noted that leaves from older plants (65 days after germination) are not able to synthesize measurable quantities of linamarin (McMahon and Sayre, 1992). The ability of roots to synthesize linamarin *in vivo* raises the issue of the relative contribution of different sites of linamarin synthesis to the overall steady-state levels of linamarin in roots. Previously, Makame et al. (1987) measured changes in linamarin content in leaves and roots of grafted plants which had high-cyanogenic variety shoots and low-cyanogenic variety roots. Their "coefficient of determination" for scion influence on root HCN content indicated there was no relationship between leaf and root HCN content. These observations are consistent with separate sites of synthesis and storage of linamarin in cassava and indicate that linamarin transport from leaves to roots is not likely to contribute substantially to the root linamarin content.

## 2.3. In vitro rates of linamarin synthesis

In order to more fully characterize the dynamics of linamarin synthesis and turnover we have measured rates of linamarin synthesis in protoplasts of leaves which would presumably be unable to transport linamarin or be influenced by sink-source relationships. We have previously shown that intact young leaves of the high-cyanogenic variety, CM 996-6, were capable of synthesizing 2.2 times more

linamarin than leaves from the low-cyanogenic variety, HMC-1 (McMahon and Sayre, 1992). However, leaves of older plants ( $\geq 65$  days), regardless of whether they were high or low cyanogenic varieties, did not synthesize detectable quantities of linamarin. In contrast to the results obtained with young leaves, we observed that leaf cell protoplasts from mature plants of the low cyanogenic variety, HMC-1, synthesized substantial amounts of linamarin at rates up to 5 times higher (in side-by-side labelings with  $0.5 \text{ mM } ^{14}\text{C}$  valine) than did protoplasts from the high cyanogenic variety, CM 996-6 (See Figure 1). At present, the basis for this inconsistency between *in vitro* and *in vivo* labeling patterns is not known, however, the disruption of sink-source relationships, age dependent differences in linamarin synthetic rates, or differences in linamarin transport may account for these results.

#### 2.4. Purification, localization, and characterization of HNL.

It has generally been accepted that linamarin is the only cyanogen present in poorly processed cassava. As previously indicated, Tylleskar, et al. (1992) demonstrated that acetone cyanohydrin was also present in substantial quantities in cassava flour. In the flour from poorly processed cassava roots acetone cyanohydrin could account for as much as 80% of the total cyanogen content. This result was surprising since it had been assumed that acetone cyanohydrin spontaneously decomposed during flour preparation. In order to understand the dynamics of cyanogen turnover in cassava we have measured the spontaneous rate of acetone cyanohydrin decomposition as a function of pH and temperature in aqueous solutions. As shown in Figure 2, acetone cyanohydrin is stable at room temperature and low pH ( $\leq 4.0$ ). However, at pHs greater than 4.0 and temperatures  $\geq 45 \text{ }^\circ\text{C}$  there is an accelerated rate of acetone cyanohydrin decomposition. In addition to spontaneous decomposition, acetone cyanohydrin can be enzymatically converted to HCN and acetone by HNL. In order to determine if the activity of HNL was rate limiting for the conversion of acetone cyanohydrin to HCN we have isolated and characterized HNL.

As shown in Table 3 and Figure 3, HNL was enriched in leaf apoplast extracts. Using apoplast extracts as our starting material, proteins were fractionated by size exclusion chromatography and passage over a concanavalin A sepharose column. HNL was then purified to apparent homogeneity by ion

exchange chromatography on a hydroxylapatite column. On average, 19 ug of apoplast protein was recovered per gram fresh weight of leaf tissue. Using the purification scheme outlined in Table 3 we obtained a ten fold enrichment in activity. The optimal activity (24 mmol/mg protein/h) was obtained at pH 5.0, as shown in Table 3. Based on the Coomassie blue staining intensities on SDS-PAGE gels, HNL and linamarase were present in nearly equal abundance in apoplast extracts (obtained in the summer). The relative HNL and linamarase activities were 8-fold and 4-fold higher in apoplastic extracts, respectively, than in whole leaf extracts. However, the quantity of linamarase changed according to season, with the percentage of linamarase decreasing substantially (approximately a 4-fold reduction) during the winter. Overall, these results are consistent with a non-symplastic (cell wall) localization for both HNL and linamarase (Mkpong et al., 1990).

We have also determined the relative activities of linamarase and HNL under conditions (pH 4.0 and 25 °C) similar to those that occur during fermentative processing. Under these conditions, the relative activities of purified HNL and linamarase were 12 mmol/mg protein/h and 2.4 mmol/mg protein/h, respectively (Mkpong, unpublished results; White and Sayre, 1992). These results, in concert with previous data indicating that the relative abundance (protein) of HNL and linamarase in whole leaf extracts (fresh weight basis) was similar, indicate that cyanogenesis is probably not limited by a disproportionate reduction in the activity of either enzyme. Another factor which could affect cyanogenesis during cassava processing is the stability of the enzymes. Therefore, we determined the stability of HNL as a function of temperature, pH, and time. We observed no loss in activity following 24 h incubations at pHs ranging from 4.0 - 7.0. As shown by Carvalho, HNL is also heat stable, i.e. there was no loss in activity following treatment at 60 °C for 45 minutes (Carvalho, 1981). These results are similar to those obtained for linamarase and are relevant to cassava processing since it is generally carried out for several days at reduced pHs (Mkpong et al., 1991; Tylleskar et al., 1992). Since both substrates (linamarin and acetone cyanohydrin) as well as the cyanogenic enzymes (linamarase and HNL) are stable at these reduced pHs and temperatures, it is suggested that inadequate conversion of linamarin to free, volatile cyanide may be due to either: 1) insufficient activity of the cyanogenic enzymes, 2) limited accessibility of the substrates to the enzymes, or 3) inhibition of cyanogenic enzyme activity. Relevant to

this last possibility Carvalho (1981) observed a 70% increase in total activity following removal of a contaminating protein fraction (by ammonium sulfate precipitation) from a partially purified HNL fraction. We also observed a 62% increase in total HNL activity in whole leaf extracts following a 45 minute, 60 °C heat treatment. This increase in activity could be due to inactivation of an inhibitor or activation of the enzyme. Studies are currently in progress to determine whether HNL activity is inhibited *in vivo* or *in vitro* by plant extracts.

### 3. Conclusions

In summary, our results indicate that linamarin is the only apparent cyanogenic glycoside synthesized from valine. Furthermore, rates of linamarin synthesis in unswollen roots were shown to be equivalent to those in leaves. We also demonstrate that both cyanogenic enzymes are apoplastic (these results are in contrast to those of Pancoro and Hughes, (1991) who have suggested that linamarase is localized in laticifers) whereas linamarin is localized in the symplast (vacuoles). These results in addition to the lack of linustatin suggest that the linamarin content of roots is probably determined by the extent of synthesis in the roots and their relative intactness. Last of all, we have characterized the production of HCN from acetone cyanohydrin. We demonstrate that under the conditions commonly found during fermentation acetone cyanohydrin is stable. The enzyme HNL, however, is also stable and active. We propose that the incomplete conversion of linamarin and acetone cyanohydrin into volatile HCN is probably due to insufficient activity of these enzymes in the processing solution.

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## References

- Arias, B. and Belloti, A.C., 1984. Aspectos ecologicos y de manejo de *Cyrtomenus bergi* Froeschner, chinche de la viruela en el cultivo de la yuca *Manihot esculenta* Crantz. CIAT Publication, Cali, Colombia.
- Balagopalan, C., Padmaja, G., Nanda, S. and Morthy, S., 1988. Cassava in Food, Feed and Industry. CRC Press, Boca Raton, Florida.
- Carvalho, J.P.C.C., 1981. Thesis for Ph.D. University of California, Davis.
- Griffith, M., Ala, D., Yang, D.S.C., Hon, W. and Moffatt, B.A., 1992. Antifreeze protein produced endogenously in winter rye leaves. *Plant Physiol.*, 100: 593-596.
- Koch, B., Nielson, V.S., Halkier, B.A., Olsen, C.E. and Møller, B.L., 1992. The biosynthesis of cyanogenic glycosides in seedlings of cassava (*Manihot esculenta* Crantz). *Arch. Biochem. Biophys.*, 292: 141-150.
- Makame, M., Akoroda, M.O., and Hahn, S.K., 1987. Effects of reciprocal stem grafts on cyanide translocation in cassava. *J. Agric. Sci. Camb.*, 109: 605-608.
- Matoh, T., Watanabe, J. and Takahishi, E., 1987. Sodium, potassium, chloride and betaine concentrations in isolated vacuoles from salt-grown *Atriplex gmelini* leaves. *Plant Physiol.*, 84: 173-177.
- McMahon, J.M. and Sayre, R.T. 1993. Differential biosynthesis and transport of linamarin in high- and low-cyanide cultivars of cassava (*Manihot esculenta* Crantz) In: W.M. Roca and A.M. Thro (Editors), Proceedings of the first international scientific meeting of the Cassava Biotechnology Network Cartagena, Colombia, 25-28 August 1992. Cali, Colombia: Centro Internacional de Agricultura Tropical, pp. 376-378.
- Mkpong, O.E., Yan, H., Chism, G. and Sayre, R.T., 1990. Purification, characterization, and localization of linamarase in cassava. *Plant Physiol.*, 93: 176-181.
- Pancoro, A. and Hughes, M.A., 1992. *In-situ* localization of cyanogenic -glucosidase (linamarase) gene expression in leaves of cassava (*Manihot esculenta* Crantz) using non-isotopic riboprobes. *The Plant Journal*, 2: 821-827.

- Saunders, J.A. and Conn, E.E., 1978. Presence of the cyanogenic glycoside dhurrin in isolated vacuoles from *Sorghum*. *Plant Physiol.*, 61: 154-157.
- Selmar, D., 1993. Transport of cyanogenic glycosides: linustatin uptake by *Hevea* cotyledons. *Planta*, 191: 191-199.
- Selmar, D., Lieberei, R. and Biehl, B., 1988. Mobilization and utilization of cyanogenic glycosides. *Plant Physiol.*, 86: 711-716.
- Szabados, L., Narvaez, J. and Roca, W.M., 1987. Techniques for isolation and culture of cassava (*Manihot esculenta* Crantz) protoplasts. Biotechnology Research Unit, CIAT, Cali, Colombia. pp. 1-4.
- Terry, M.E., and Bonner, B.A., 1980. An examination of centrifugation as a method of extracting an extracellular solution from peas. and its use for the study of indoleacetic acid-induced growth. *Plant Physiol.*, 66: 321-325.
- Tylleskar, T., Cooke, R.D., Banea, M., Poulter, N.H., Bikangi, N., and Rosling, H., 1992. Cassava cyanogens and konzo, an upper motorneuron disease found in Africa. *Lancet*, 339: 208-211.
- White, W. L. B., and Sayre, R.T., 1993. Partial purification and characterization of hydroxynitrile lyase from cassava. In: W.M. Roca and A.M. Thro (Editors), Proceedings of the first international scientific meeting of the Cassava Biotechnology Network Cartagena, Colombia, 25-28 August 1992. Cali, Colombia: Centro Internacional de Agricultura Tropical, pp. 379-383.

Table 1. HCN Equivalents in Protoplasts and Vacuoles

Source	$\mu\text{mol HCN}/10^6$ cells or vacuoles	Standard Deviation
<i>Cassava</i>		
Protoplasts	0.025	(0.012)
Vacuoles	0.162	(0.077)
<i>Sorghum</i>		
Protoplasts	1.09	(0.19)
Vacuoles	1.13	(0.45)

Protoplasts were isolated overnight using a procedure developed by Szabados et al. (1987). Vacuoles were obtained from protoplasts by incubation with 1 mM CHAPS and were fractionated over a linear Ficoll gradient. Water-soluble compounds were extracted from cells or vacuoles in boiling 80% methanol followed by extraction with chloroform (Brimer and Dalgaard, 1984). Extracts were assayed for HCN release after incubation with linamarase at 37 °C for one hour. The values obtained are the average of three experiments, using low-cyanogenic variety HMC-1. The HCN data for *Sorghum* protoplasts and vacuoles are from Saunders and Conn (1978).

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Table 2.  $^{14}\text{C}$ -Linamarin Synthesis in Roots and Leaves of High and Low Cyanogenic Varieties of Cassava<sup>a</sup>

		High Cyanogen	Low Cyanogen
		$\mu\text{mol/gfw/hr}$	$\mu\text{mol/gfw/hr}$
Leaf	greenhouse	$9.84 \times 10^{-4}$	$4.5 \times 10^{-4}$
Root	<i>in vitro</i>	ND	$9.0 \times 10^{-4}$
	greenhouse	ND	$1.10 \times 10^{-3}$

<sup>a</sup> High CN= CM996-6; Low CN= HMC-1; Low CN *in vitro*= MCol 2215

Tissues were incubated in 0.5 mM valine containing  $^{14}\text{C}$ -valine. The fifth leaves from the apical meristem of 49-day-old plants were labeled while still attached to the plants. Roots were removed from plants, sliced into 4 cm pieces and incubated with  $^{14}\text{C}$ -valine under constant agitation. Cyanogenic glycosides were extracted using the method of Brimer and Dalgaard (1984). Water-soluble compounds were separated using thin-layer chromatography and the amount of  $^{14}\text{C}$ -linamarin was quantified.

Table 3. Cassava Hydroxynitrile Lyase Purification

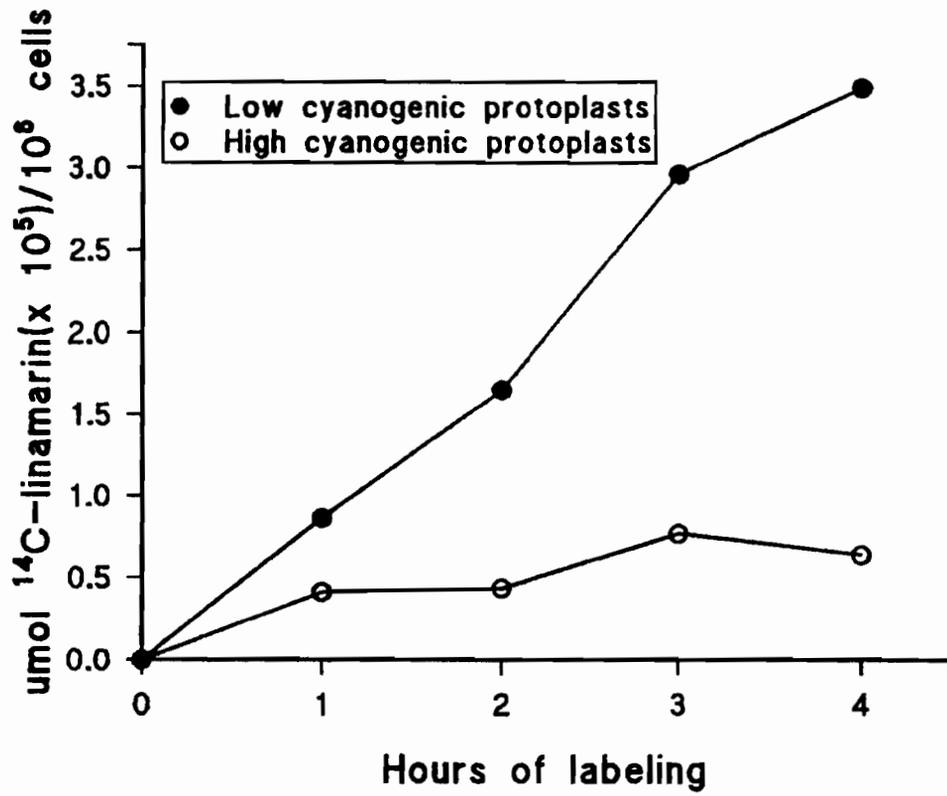
Fraction	Total Protein	Total Activity	Specific Activity	Purification Fold
	<i>mg</i>	<i>mmol/h</i>	<i>mmol/mg/h</i>	
Apoplast extract	1.580	3.8	2.4	1
Sephacryl S-200	0.553	3.7	6.7	2.8
Con A Sepharose	0.102	1.3	13	5.5
Hydroxylapatite	0.035	0.85	24	10

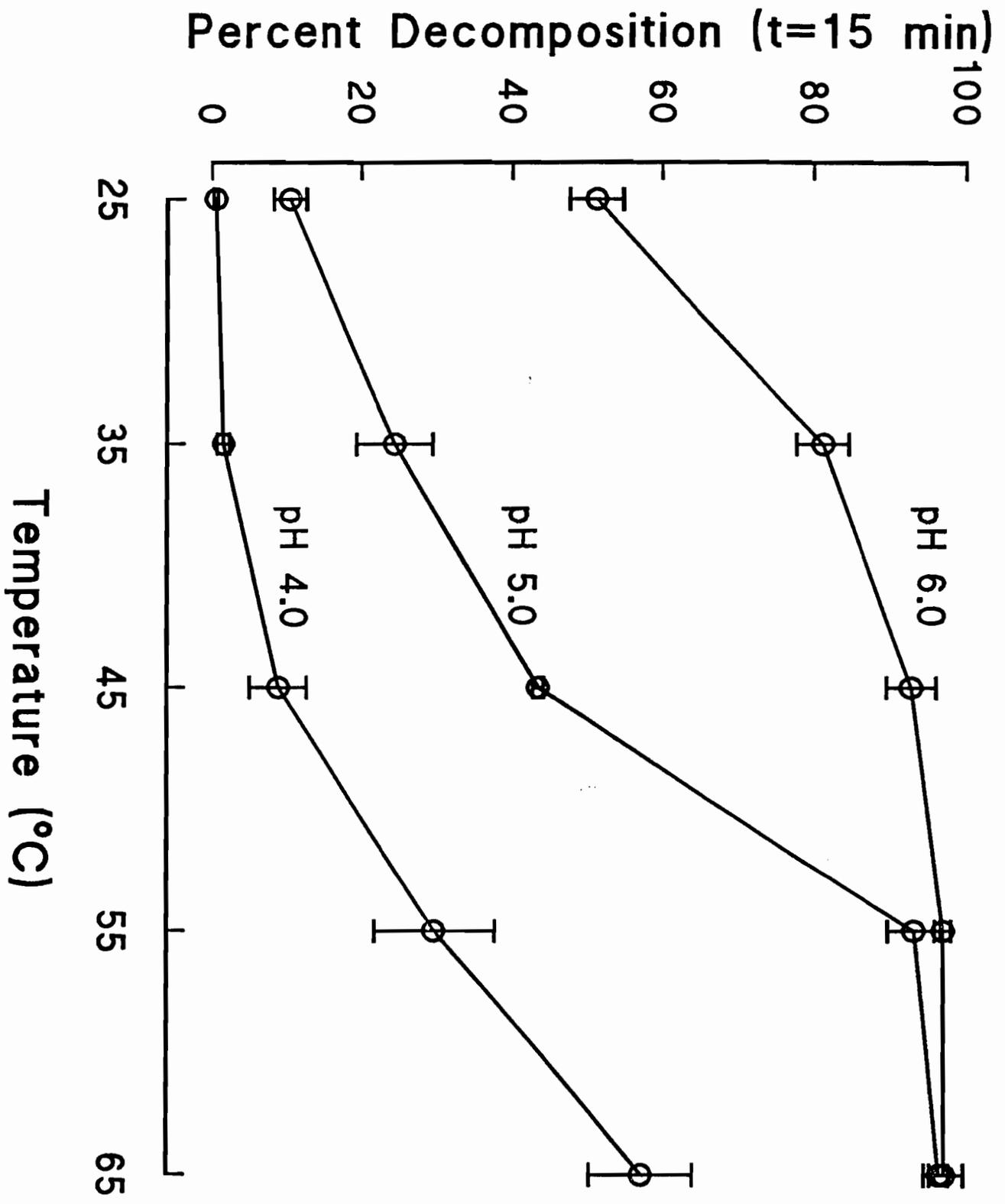
Protein was quantified by Bradford method. A Spectroquant kit was used for HCN determination. Reactions were in 50 mM sodium phosphate buffer, pH 5.0, with 28 mM acetone cyanohydrin at room temperature for 15 minutes. The apoplast extraction procedure was modified from Griffith et al. (1992) and Terry and Bonner (1980).

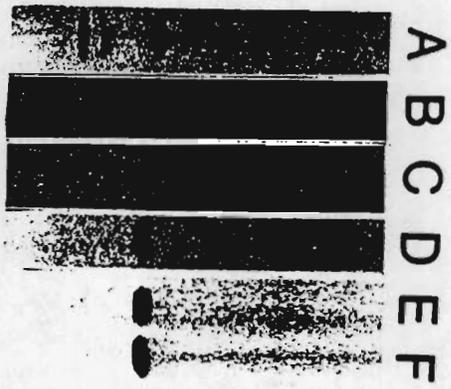
Figure 1 - Time course of  $^{14}\text{C}$ -linamarin synthesis in protoplasts of high- (CM996-6) and low-cyanogenic (HMC-1) cultivars of cassava. Protoplasts were labeled in 0.5 mM valine ( $3\mu\text{Ci}/\mu\text{mol } ^{14}\text{C}$ -valine) for 4 hours. Aliquots were removed at one-hour time intervals, and water soluble compounds were extracted using the procedure of Brimer and Dalgaard (1984). Labeled products were separated by thin-layer chromatography and analyzed for radioactive content using a phosphorimaging screen.

Figure 2 - Temperature and pH dependent degradation of acetone cyanohydrin. Each point is the average of 2 or 3 replicates. HCN was assayed using a Spectroquant kit. The initial acetone cyanohydrin concentration was 11  $\mu\text{M}$  in 50mM sodium phosphate buffers.

Figure 3 - SDS-PAGE of purified HNL from cassava. (A) protein standards; (B) 3  $\mu\text{g}$  of whole leaf crude extract; (C) 0.25  $\mu\text{g}$  of apoplast extract; (D) 2.1  $\mu\text{g}$  of Sephacryl column fraction; (E) 1  $\mu\text{g}$  of Con A Sepharose column fraction; and (F) 0.8  $\mu\text{g}$  of purified HNL from hydroxylapatite column fraction. The upper arrow points to linamarase (65 kD) and the lower to HNL ( $25 \pm 5$  kD).







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## DIFFERENTIAL INHIBITION OF TRANSIENT GENE EXPRESSION IN CASSAVA ROOT AND LEAF TISSUES

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In order to determine whether the cauliflower mosaic virus 35S promoter could drive gene expression in different cassava tissues, we introduced a plasmid containing this promoter fused to the  $\beta$ -glucuronidase reporter gene into cassava tissues by particle inflow injection. We observed several hundred blue spots or localized transformed regions expressing  $\beta$ -glucuronidase activity in leaves but virtually none in roots. A possible explanation for this differential expression is that foreign DNA could be degraded by endogenous nucleases of the roots before it is expressed. Incubation of phage DNA with root extracts resulted in complete degradation of the DNA in as little as 10 minutes, unlike incubation with leaf extracts. Comparative analysis of the DNase activity in roots versus leaves indicates that there is substantially higher DNase activity in the root tissue than leaf tissue. We suggest that the differential abundance of nuclease activity in the two tissues accounts for the tissue specific differences in transient DNA transformation and gene expression in cassava.

### INTRODUCTION

It is estimated that over 300 million people in tropical countries subsist on a cassava based diet, however, cassava roots have a low protein content and contain cyanogenic glycosides which can cause neurological disorders (3,9). These and other features may be amenable to modification via genetic transformation but first it is necessary to identify strong root specific promoters which can be used to drive the expression of introduced genetic material.

In order to identify such promoters, we introduced plasmids containing various promoters fused to the bacterial  $\beta$ -glucuronidase reporter gene (GUS) into cassava tissues via a particle inflow gun. Using a cauliflower mosaic virus 35S (CaMV 35S) promoter gene fusion, we routinely observed significant differences in the number of transiently transformed regions (blue spots) expressing GUS activity in cassava leaf and root tissues. Comparative analyses of the levels of DNase activity and  $\beta$ -glucuronidase inhibitor(s) in roots and leaves indicated that there were significant differences in these activities between different tissues.

Based on these observations, we suggest that the tissue specific differences in the number of transforming events may be due either to differences in the ability to deliver DNA intact to the nuclei or to inhibition of  $\beta$ -glucuronidase activity.

## MATERIALS AND METHODS

### Plant Material

Seeds and/or stem cuttings of cassava (Manihot esculenta Crantz) varieties Mcol 2215 and Mven 25 were obtained from the International Center for Tropical Agriculture (CIAT), Cali, Colombia. Plants were grown under greenhouse or in vitro conditions (10).

### Transient DNA Transformation

Plasmid pBI221 (Clontech), containing the CaMV 35S promoter fused to the  $\beta$ -glucuronidase gene and a NOS terminator sequence or plasmid pBinGSGUS containing the soybean glutamine synthetase promoter fused to the GUS gene (5) was precipitated onto DNA coated tungsten particles according to the procedure of Finer and McMullen (4). Tungsten particles (2.5  $\mu$ l) were shot into cassava leaf or root tissues using a particle inflow gun at a helium pressure of 80 psi (4,7). In each case, an equal area (3 cm circle) of plant material was shot in a Petri dish containing standard culture medium for cassava (10).

Transient expression of GUS activity was visualized by staining with X-Gluc, according to the procedures described by Jefferson (7).

### Crude Protein Extracts

Cassava root and leaf proteins were extracted from 1 gm of in vitro (sterile) grown plant tissue. The plant tissue was ground to a fine powder in liquid nitrogen in 3 ml of 50 mM HEPES, pH 7.5, 5.0 mM  $MgCl_2$ , and centrifuged twice at 23,000 x G for 15 min to remove cell debris (1). The supernatant was stored at -80 °C until use. Protein concentrations were determined by the method of Bradford (2).

### DNase Activity

One  $\mu$ g of lambda phage DNA was incubated for various time intervals with cassava crude protein extract in 25  $\mu$ l final volume of buffer containing 2.0 mM  $MgCl_2$  and 25 mM HEPES pH 7.5 at room temperature. The DNA was then electrophoresed in a 1.2% (w/v) TBE agarose gel containing ethidium bromide (0.5  $\mu$ g/ml) and photographed using a UV transilluminator.

DNase activity was also quantified by determination of precipitable DNA following incubation with crude protein extracts (8). Random primer  $^{32}P$ -labelled DNA (30 ng) was incubated with 0.3  $\mu$ g of crude cassava protein extract in 2 mM  $MgCl_2$  and 25 mM HEPES, pH 7.5 at room temperature in a final volume of 25  $\mu$ l. The reaction was stopped after 30 min incubation by the addition of 100  $\mu$ l of a solution containing salmon sperm DNA (500  $\mu$ g/ml) and 20 mM EDTA, pH 8.0, followed by addition of 14  $\mu$ l of 100% (w/v) TCA. The solution was then incubated on ice for 15

min, pelleted at 13,500 x G for 15 min and washed with 1 ml of 10% (w/v) TCA followed by 1 ml of 70% (v/v) ethanol. The precipitated DNA was resuspended in 400  $\mu$ l of water for quantification by liquid scintillation counting (1).

### **$\beta$ -glucuronidase Activity**

Bacterial  $\beta$ -glucuronidase (Sigma) was incubated with or without cassava tissue extracts in a reaction mixture containing: 500  $\mu$ l of 75 mM potassium phosphate buffer, pH 6.8 plus 0.1% (w/v) BSA; 250  $\mu$ l of 3.0 mM p-nitrophenol  $\beta$ -D glucuronide in 50 mM HEPES, pH 7.5; 5  $\mu$ l (4 units) of  $\beta$ -glucuronidase and various amounts of tissue extracts in a final volume of 1,500  $\mu$ l. Following 30 min incubation at 37 °C the reactions were stopped by addition of 5 ml of 0.2 M glycine, pH 10.4 and the nitrophenol produced was quantified spectrophotometrically at 400 nm. Control blanks were prepared the same way except that the glycine buffer was added prior to addition of the  $\beta$ -glucuronidase solution. In some assays tissue extract was added before addition of the substrate. The tissue extract/ $\beta$ -glucuronidase mixtures were then pre-incubated for various times, with and without PMSF, prior to initiation of the assay.

## **RESULTS AND DISCUSSION**

In order to determine whether the CaMV 35S promoter would drive gene expression in different cassava tissues we shot a CaMV 35S-GUS reporter gene construct into cassava and quantified the number of regions transiently expressing  $\beta$ -glucuronidase activity. We observed numerous localized regions of GUS activity in leaf tissue but virtually none in roots. Similarly, using a soybean, root specific promoter from the glutamine synthetase gene (5) we observed no expression of  $\beta$ -glucuronidase activity in roots (Note: no GUS activity was observed in leaves either using this promoter). One possible explanation for why root specific transient gene expression may be reduced is degradation of the introduced DNA by endogenous nuclease activity (6). In order to determine whether there were tissue specific differences in total nuclease activity, crude tissue extracts were incubated with lambda DNA for various time intervals and DNA integrity was analyzed. In the presence of 0.2  $\mu$ g of root protein lambda DNA was substantially degraded in as little as 10 min, whereas there was little evidence of DNA degradation in the presence of 20 fold higher levels of leaf protein (4  $\mu$ g) even after 2 hours of incubation.

In order to quantify the level of DNase activity, we quantified the amount of precipitable  $^{32}$ P-labeled DNA following incubation with crude tissue extracts. It was found that 26% of the labeled DNA was degraded when incubated with 0.3  $\mu$ g of root crude protein extract. This level of DNase activity was 3.25 times higher than that found in leaf crude extract (1). This difference was even more apparent when we used reduced amounts of extracts (0.15  $\mu$ g of protein). It is apparent that the DNase activity in roots can cause substantial damage to high molecular weight DNA (Note: similar effects were observed with pBI221 and pBinGSGUS plasmid DNA). At present, the cellular location of the DNase activity is not known, however, preliminary

studies indicate that the pH optimum for this activity is between 6.0 and 6.5, suggesting that it may not be cytoplasmic.

We also investigated the possibility that the lack of GUS expression in roots could be due to the presence of inhibitors of GUS or proteases. The addition of root extract equivalent to 13% of the total assay volume inhibited GUS activity by 34%. Expressed on a protein basis, 20  $\mu\text{g}$  of root extract was 5 fold more effective in inhibiting  $\beta$ -glucuronidase activity than was an equivalent amount of leaf extract. Since the protein concentration of root extracts was generally several times less than that of leaf extracts, expression of GUS activity on a volume basis is probably more reflective of the *in vivo* conditions than is expression on the basis of protein concentration. In addition, we also compared the effect of pre-incubation of  $\beta$ -glucuronidase with tissue extracts on GUS activity. We observed no differences in GUS activity between 0 and 60 min pre-incubation with root extract either in the presence or absence of PMSF. These results suggest that GUS inhibition is rapid and that proteolytic (serine type) activity is probably not responsible for the inhibition of GUS activity by root extracts.

In conclusion, these results suggest that transient gene expression assays, particularly those using the B-glucuronidase reporter gene, may not be feasible for analysis of gene expression in cassava root tissue.

#### REFERENCES

1. Arias-Garzon DI, Sayre RT (1992) Tissue specific inhibition of transient gene expression in cassava (Manihot esculenta Crantz). Submitted to Plant Physiology.
2. Bradford M (1976) A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-drug binding. Anal Biochem 72: 248-254.
3. Cock JH (1982) Cassava: A basic energy source in the tropics. Science Vol 218: 755-762.
4. Finer, JJ, Vain P., Jones MW, McMullen, MD (1992) Development of a particle inflow gun for delivery of DNA to plant cells. Plant Cell Reports (in press).
5. Guo-Hua M, Hirel B, Marsolier MC, Ridge RW, Verma DP (1991) Ammonia-Regulated expression of Soybean gene encoding cytosolic Glutamine Synthetase in transgenic Lotus corniculatus. The Plant Cell, 3: 11-22.
6. Hughes B, White F, Smith M (1979) Fate of bacterial plasmid DNA during uptake by Barley and Tobacco protoplasts: II Protection by Poly-L-Omithine.

7. Jefferson RA (1987) Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Molecular Biology Reporter* 5: 387-405.
8. Maniatis T, Fritsch EF, Sambrock J (1982) *Molecular Cloning, A laboratory Manual*. Cold Spring Harbor Laboratory, New York.
9. Mkpong O, Yan H, Chism G, Sayre RT (1990) Purification, Characterization, and localization of linamarase in cassava. *Plant Physiol.* 93: 176-181.
10. Roca WM (1984) Cassava. In WR Sharp, DA Evans, PV Ammirato, Y Yamada, eds. *Handbook of plant cell culture, Vol. 2 Crop Species*. MacMillan Publishing, New York, pp 269-301.



## Tissue specific inhibition of transient gene expression in cassava (*Manihot esculenta* Crantz)†

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### Abstract

We have surveyed a variety of promoter-reporter gene constructs and transient transformation protocols to identify strong root specific promoters in the tropical crop plant cassava (*Manihot esculenta*, Crantz). Using a cauliflower mosaic virus 35S promoter, we observed several hundred localized regions which expressed  $\beta$ -glucuronidase activity in cassava leaves but virtually none in roots. Similarly, a low frequency of transient gene expression was observed in roots when bombarded with a soybean root-specific glutamine synthetase promoter- $\beta$ -glucuronidase reporter gene. Another reporter gene (luciferase) fused to the CaMV 35S promoter gave similar results indicating that the low frequency of transient gene expression in roots was not a consequence of the particular reporter gene used. In our attempts to resolve the basis for the low frequency of transient gene expression in roots, we determined that root tissues have high levels of DNase activity. It is postulated that the high levels of DNase activity compromises the integrity of the transforming DNA resulting in reduced transformation efficiency.

**Key words:** Transient gene expression;  $\beta$ -Glucuronidase; Luciferase; DNase; Cassava

### 1. Introduction

Cassava (*Manihot esculenta*) is a perennial shrub grown throughout the lowland tropics for its starchy thickened roots [1]. It is estimated that over 300 million people subsist on a cassava-based diet [1,2]. Cassava roots, however, have a low protein content and contain cyanogenic glycosides which can cause neurological disorders [3–5]. These and other aspects of the nutritional quality of cassava roots are potentially amenable to genetic modifica-

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**Abbreviations:** BSA, bovine serum albumin; CaMV, cauliflower mosaic virus; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GUS,  $\beta$ -glucuronidase; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; LUC, luciferase; MS, Murashige and Skoog medium; NOS, nopaline synthetase; PMSF, phenylmethylsulfonyl fluoride; TBE pH 7.5–7.8, 89 mM Tris, 89 mM borate, 1 mM EDTA buffer pH 8.0; TCA, trichloroacetic acid; tricine, *N*-tris-[hydroxymethyl]methyl glycine; X-gluc, 5-bromo-4-chloro-3-indolyl-B-D-glucuronic acid.

tion via stable introduction and expression of recombinant DNA molecules. In order to genetically alter the nutritional quality of cassava roots, it is necessary to identify strong root specific gene promoters which can be used to direct the expression of introduced DNA sequences.

The cauliflower mosaic virus 35S (CaMV 35S) promoter has been shown to direct high levels of gene expression in a variety of plant tissues including tobacco root tissue and cassava leaf tissue [6–9]. In order to determine whether the CaMV 35S promoter or a root specific promoter would direct gene expression in cassava root tissue, we bombarded cassava roots with various promoter-reporter gene constructs and assayed for transient gene expression. Regardless of the transforming DNA used, we routinely observed significantly fewer transformed regions or lower levels of reporter gene activity in root tissues than in leaves. The levels or frequency of reporter gene activity were, in all but one case, inversely correlated with the level of DNase activity. We propose that low frequencies of transient transformation may be due to degradation of transforming DNA associated with DNase activity.

## 2. Materials and methods

### 2.1. Plant material

Seeds and/or stem cuttings of cassava (*Manihot esculenta* Crantz) varieties Mcol 2215 and Mven 25 were obtained from Dr. Clair Hershey at the International Center for Tropical Agriculture (CIAT), Cali, Colombia. Plants were grown under greenhouse or in vitro conditions. Shoot apical meristems were grown on standard propagation medium for cassava [10] containing MS salts [11], 1 mg/l thiamine-HCl, 100 mg/l *m*-inositol, 2% (w/v) sucrose, 0.02 mg/l naphthaleneacetic acid, 0.04 mg/l benzylamino-purine and 0.05 mg/l gibberellic acid; the medium was solidified with 0.7% (w/v) Bacto-agar. Shoot cultures were maintained under a 12 h/day photoperiod, at 27°C and a light intensity of  $50 \mu\text{E m}^{-2} \text{s}^{-1}$  (300–700 nm).

Soybean seeds (c.v. Williams) (kindly provided by Dr. Dietz Bauer, The Ohio State University) were germinated under the same conditions as for cassava shoot cultures.

### 2.2. Transient DNA transformation

Cassava and soybean tissues were transiently transformed using either of three plasmids: pBI221 (Clontech), containing the CaMV 35S promoter fused to the  $\beta$ -glucuronidase gene and a NOS 3' terminator sequence; pBinGSGUS, containing the soybean root-specific glutamine synthetase promoter fused to the GUS gene [12], or pDO432 containing the CaMV 35S RNA promoter-luciferase-NOS 3' terminator sequence in pUC 19 [13]. Plasmid DNA was precipitated onto tungsten or gold particles according to the procedure of Finer and McMullen [14]. DNA-coated particles were introduced into cassava and soybean leaf or root tissues using either a particle inflow gun at a helium pressure of 80 psi or a biolistic PDS-1000 particle delivery system (DuPont) with either gold or tungsten particles (M10 or M17) [14,15]. In general, in vitro grown sterile leaves and roots were bombarded to avoid possible complications due to the expression of GUS activity from contaminating microorganisms. In each case, an equal area (3 cm circle) of plant material was shot in a Petri dish containing standard medium for cassava tissue cultures [10].

### 2.3. In situ GUS expression

Transient expression of GUS activity was visualized by staining with the chromogenic substrate X-gluc [16]. Following bombardment, the explants were incubated overnight in Petri dishes containing MS basal medium at room temperature in complete darkness. The explants were then placed in 400  $\mu\text{l}$  of a solution containing 50  $\mu\text{g}$  X-gluc (Research Organics) in 10 mM EDTA, pH 8.0; 100 mM sodium phosphate, pH 7.0; 5 mM potassium ferrocyanide; 5 mM potassium ferricyanide and 1  $\mu\text{l}$  Triton X-100 for 16–24 h at 37°C to visualize GUS activity [16].

### 2.4. Crude extracts

Cassava or soybean soluble fractions were extracted from 0.8 g of in vitro (sterile) grown plant tissue. The plant tissue was ground to a fine powder in liquid nitrogen, transferred to 3 ml of 50 mM HEPES pH 7.5, 5 mM  $\text{MgCl}_2$ , and the solution centrifuged twice at  $23\,000 \times g$  for 15 min to remove cell debris. The supernatant fraction was

stored at  $-80^{\circ}\text{C}$  until used. Protein concentrations were determined by the method of Bradford [17].

### 2.5. $\beta$ -Glucuronidase activity

Bacterial  $\beta$ -glucuronidase (Sigma) was assayed in a reaction mixture containing  $500\ \mu\text{l}$  of  $75\ \text{mM}$  potassium phosphate buffer, pH 6.8 plus  $0.1\%$  (w/v) BSA;  $250\ \mu\text{l}$  of  $3.0\ \text{mM}$  *p*-nitrophenol  $\beta$ -D-glucuronide in  $50\ \text{mM}$  HEPES, pH 7.5; in the absence or presence of various amounts of tissue extracts to give a final volume of  $1.5\ \text{ml}$ . The reaction was started by the addition of  $5\ \mu\text{l}$  (4 units) of the enzyme. Following 30 min incubation at  $37^{\circ}\text{C}$ , the reactions were stopped by addition of  $5\ \text{ml}$  of  $0.2\ \text{M}$  glycine, pH 10.4 and the nitrophenol produced was quantified spectrophotometrically at  $400\ \text{nm}$ . Control blanks were prepared the same way except that the glycine stop buffer was added prior to addition of the  $\beta$ -glucuronidase solution. In some assays the tissue extract/ $\beta$ -glucuronidase mixtures were pre-incubated for various times, with and without  $0.1\ \text{mM}$  PMSF, prior to initiation of the assay by addition of substrate ( $\beta$ -D-glucuronide).

### 2.6. Luciferase enzyme assay

Luciferase activity was measured using cassava crude tissue extracts with a luciferase assay kit (Promega E1500). After the plant tissues were bombarded, they were incubated overnight in Petri dishes containing MS basal medium at  $27^{\circ}\text{C}$  in complete darkness. The tissues ( $0.8\ \text{g}$ ) were then ground in liquid nitrogen in  $3\ \text{ml}$  of cell culture lysis reagent (containing  $25\ \text{mM}$  Tris-phosphate, pH 7.8;  $2\ \text{mM}$  DTT,  $2\ \text{mM}$  1,2 diaminocyclohexane-*N,N,N',N'*-tetraacetic acid;  $10\%$  (v/v) glycerol and  $1\%$  (v/v) Triton X-100), centrifuged at  $13\ 000 \times g$  for  $10\ \text{min}$  at  $4^{\circ}\text{C}$  and the supernatant fraction filtered through sterile Miracloth to remove pelleted cell debris. Luciferase activity was measured by introducing  $50\ \mu\text{l}$  of the crude tissue extract into a glass tube containing  $100\ \mu\text{l}$  of luciferase assay buffer ( $20\ \text{mM}$  tricine, pH 7.8;  $1.07\ \text{mM}$   $(\text{MgCO}_3)_4$ ,  $\text{Mg}(\text{OH})_2 \cdot 5\text{H}_2\text{O}$ ;  $2.67\ \text{mM}$   $\text{MgSO}_4$ ;  $0.1\ \text{mM}$  EDTA;  $33.3\ \text{mM}$  DTT;  $270\ \mu\text{M}$  coenzyme A;  $470\ \mu\text{M}$  luciferin and  $530\ \mu\text{M}$  ATP) and immediately assayed for luminescence using a Monolight 350 luminometer (Analytical Lumin-

escence Laboratory, San Diego, CA) with an integration time of  $10\ \text{s}$ .

Assays for luciferase inhibitor activity were made in a reaction mixture containing  $24\ \text{ng}/\mu\text{l}$  of firefly luciferase (Sigma) dissolved in glycine buffer ( $1\ \text{M}$  pH 7.7), plus various amounts of crude cassava extract in a final volume of  $200\ \mu\text{l}$ . The reaction was started by the addition of  $1\ \mu\text{l}$  of luciferase assay buffer which contains  $470\ \mu\text{M}$  luciferin and  $530\ \mu\text{M}$  ATP. Luminescence was determined using the same conditions as in the luciferase enzyme activity.

### 2.7. DNase activity

Lambda phage DNA ( $1\ \mu\text{g}$ ) was incubated for various time intervals with cassava or soybean crude tissue extract in  $25\ \mu\text{l}$  final volume of buffer containing  $2.0\ \text{mM}$   $\text{MgCl}_2$  and  $25\ \text{mM}$  HEPES, pH 7.5 at room temperature. The DNA was then subjected to electrophoresis in a  $1.2\%$  (w/v) TBE agarose gel containing ethidium bromide ( $0.5\ \mu\text{g}/\text{ml}$ ) and photographed using a UV trans-illuminator.

DNase activity was also quantified by determination of precipitable DNA following incubation with crude cassava tissue extracts [18]. Random primer  $^{32}\text{P}$ -labelled DNA ( $30\ \text{ng}$ ) was incubated with  $0.3\ \mu\text{g}$  of crude cassava tissue extract in  $2\ \text{mM}$   $\text{MgCl}_2$  and  $25\ \text{mM}$  HEPES, pH 7.5 at room temperature in a final volume of  $25\ \mu\text{l}$ . The reaction was stopped after 30 min incubation by the addition of  $100\ \mu\text{l}$  of a solution containing salmon sperm DNA ( $500\ \mu\text{g}/\text{ml}$ ) and  $20\ \text{mM}$  EDTA, pH 8.0, followed by addition of  $14\ \mu\text{l}$  of  $100\%$  (w/v) TCA. The solution was then incubated on ice for  $15\ \text{min}$ , pelleted at  $13\ 500 \times g$  for  $15\ \text{min}$  and washed with  $1\ \text{ml}$  of  $10\%$  (w/v) TCA followed by  $1\ \text{ml}$  of  $70\%$  (v/v) ethanol. The precipitated DNA was resuspended in  $400\ \mu\text{l}$  of water for quantification by liquid scintillation counting.

## 3. Results and discussion

Although  $\beta$ -glucuronidase (GUS) activity can be transiently expressed in cassava leaf tissue using either the CaMV 35S promoter or the ubiquitin 1 gene promoter from *Arabidopsis thaliana* [8], it is

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unclear whether these or other promoters would direct gene expression in cassava roots. In order to determine whether the CaMV 35S promoter would permit gene expression in different cassava tissues we introduced, via particle bombardment, a CaMV 35S-GUS reporter gene construct (pBI221) into cassava and quantified the number of regions transiently expressing  $\beta$ -glucuronidase activity. Various transformation procedures were tested in order to obtain optimal transient transformation frequencies, including (i) the type of gun used to deliver the DNA coated particles (PDS-1000 particle delivery system (Dupont) using gun powder as an accelerant, or a particle inflow helium gun); (ii) the type of DNA-coated particles used (1.0  $\mu$  gold particles vs. 0.7  $\mu$  or 1.1  $\mu$  tungsten particles); (iii) the distance between the gun orifice and tissue and (iv) the type of tissue bombarded including, growth chamber grown and in vitro grown leaves and primary roots sectioned in various manners. We observed the highest transient transformation frequencies in leaves and roots when bombarded with the helium-driven particle inflow gun using M-17 tungsten particles (1.1  $\mu$ ) (Table 1, and data not shown). It is noteworthy that the conditions which we determined to be optimal for transient transformation of cassava were quite similar to those found for other plant species [19].

There were, however, significant differences in the transformation frequency (GUS expressing

spots/unit area) between leaf and root tissues. Using the CaMV 35S-GUS reporter gene construct (pBI221), we observed several hundred (230/7 cm<sup>2</sup>) localized regions expressing GUS activity in transiently-transformed cassava leaf tissue but virtually none in roots ( $\leq 1/7$  cm<sup>2</sup>) (Fig. 1, Table 1). In only one case was GUS activity detected in cassava root tissue. The intensity of the GUS product in the transformed root tissue was, however, similar to that observed in leaves.

Since root tissues were bombarded under a variety of conditions, it is unlikely that tissue specific differences in cell number or physical barriers to particle delivery account for the differences in transformation frequency between roots and leaves. Furthermore, since the root tissues contained meristematic (primary cell wall, non-vacuolated) and fully expanded (secondary cell wall, vacuolated) cells it is also unlikely that the developmental or metabolic state of the root tissue precluded transient expression of introduced DNA.

It is generally assumed that tissue specific differences in transient gene expression reflect differences in promoter specificity and/or transcriptional control factors [6,17,20]. In order to determine whether the lack of GUS expression in cassava roots simply reflects an inability of the CaMV 35S promoter to direct transcription, we bombarded cassava with a root-specific glutamine

Table 1  
Number of transient GUS expressing spots in cassava leaf and root tissues following transformation with pBI221 using the helium particle inflow gun [16] under a variety of conditions

Gun level	Tissue	Growth	Average number of blue spots	S.D.
2	Leaf	in vitro	110	25.8
3	Leaf	in vitro	230	59.3
2	Root	in vitro	0	
3	Root	in vitro	0.16	0.41
2	Root (xs)	soil	0	
3	Root (xs)	soil	0	
2	Root (ls)	soil	0	
3	Root (ls)	soil	0	

Gun level 2 is 12 cm and level 3 is 10 cm from the gun opening. xs, cross section; ls, longitudinal section. Values are the average of 6 experiments using equal areas of tissue.

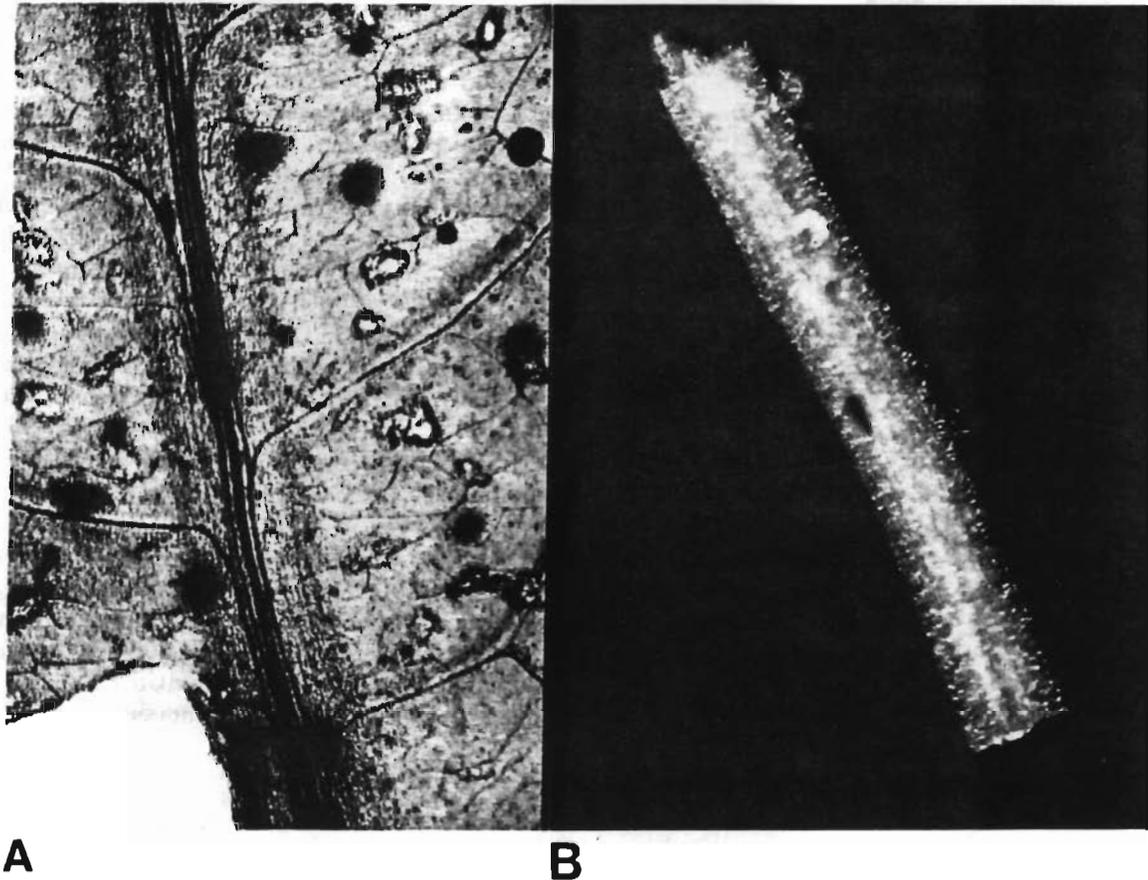


Fig. 1. Histochemical localization of GUS activity in *in vitro* grown cassava leaves (A) and roots (B) after bombardment with a CaMV 35S-GUS construct (pBI221). The blue spots are the result of the reaction catalyzed by GUS on the substrate (X-gluc), producing an indigo dye precipitate at the site of enzymatic activity.

synthetase promoter-GUS plasmid (pBinGSGUS). We observed no GUS expression in either cassava leaves (as expected) or roots using pBinGSGUS. In order to verify that we could transiently transform root tissue we also bombarded soybean roots with the root-specific glutamine synthetase promoter-GUS plasmid (pBinGSGUS) as well as the CaMV 35S-GUS plasmid (pBI221). Both plasmids were expressed at equal frequencies in soybean roots suggesting that the efficiency of transient transformation in soybean roots was not determined by the promoter type used to drive GUS expression (Table 2).

Transient gene expression patterns in cassava and soybean tissues were similar in one respect. The frequency of CaMV 35S-GUS expression was

Table 2

Transient expression of the GUS activity driven by the CaMV 35S (pBI221) and glutamine synthetase (pBinGSGUS) promoters in cassava and soybean tissues

Tissue	Plasmid	Average number of blue spots	S.D.
Cassava leaves	pBI221	162	18.24
Cassava leaves	pBinGSGUS	0	
Cassava roots	pBI221	0	
Cassava roots	pBinGSGUS	0	
Soybean leaves	pBI221	250	50.49
Soybean leaves	pBinGSGUS	0	
Soybean roots	pBI221	12	3.65
Soybean roots	pBinGSGUS	10	4.83

All shots were made at 10 cm from the gun opening. Values are the average of 4 experiments using equal areas of tissue.

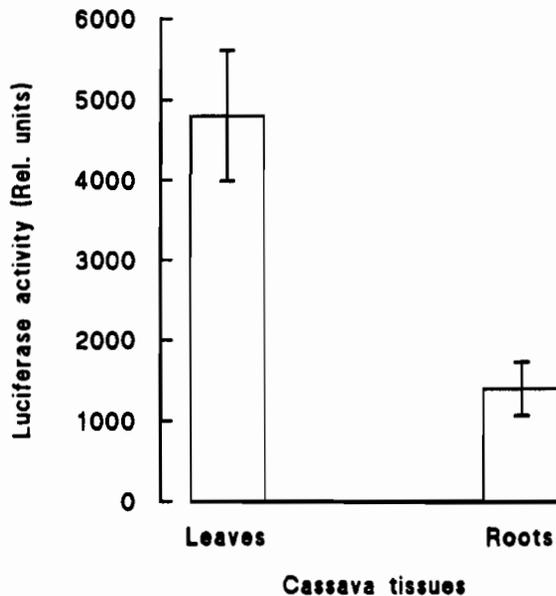


Fig. 2. Luciferase activity in transiently-transformed (pDO432) cassava tissues. Results are the average of 6 experiments. The flags indicate S.D. See Materials and methods for procedures. Relative units, light units/mg of crude protein/h).

significantly higher in leaves than in roots for both species (Table 2). Thus it appears that in both species the efficiency of root transient transformation is low relative to leaves. One possible reason for the reduced frequency of transient gene expression in roots is inactivation of GUS activity. To test this possibility we bombarded cassava tissue with a luciferase reporter gene linked to the CaMV 35S promoter. The luciferase gene has been shown to be a sensitive reporter gene in a variety of animal and plant cells [13,21,22], and would presumably be insensitive to possible GUS enzyme inhibitors. As shown in Fig. 2, luciferase activity was substantially higher (10-fold) in leaf extracts than in root extracts, consistent with the results obtained for the frequency of GUS expression in the two tissue types.

Since transient transformation of cassava roots with the CaMV 35S-luciferase gave higher apparent transformation efficiency than that obtained with the CaMV 35S-GUS plasmid we explored the possibility that GUS activity in roots was reduced due to enzyme inhibitors or to proteolysis. To test

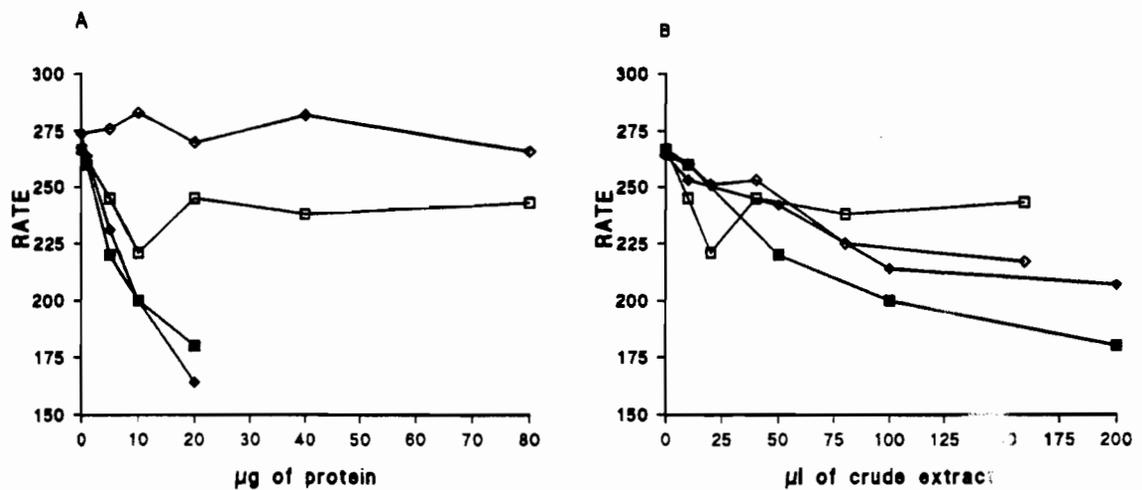


Fig. 3. Inhibition of in vitro GUS activity by cassava and soybean tissue extracts. Results are the average of 4 experiments from 2 separate tissue extractions expressed on the basis of quantity of protein (A) of tissue extract added or volume (B) of tissue extract added. Total volume of assay was 1500 µl. Cassava roots (■), cassava leaves (□), soybean roots (◆), soybean leaves (◇). Rate: mmol nitrophenol mg  $\beta$ -glucuronidase/h).

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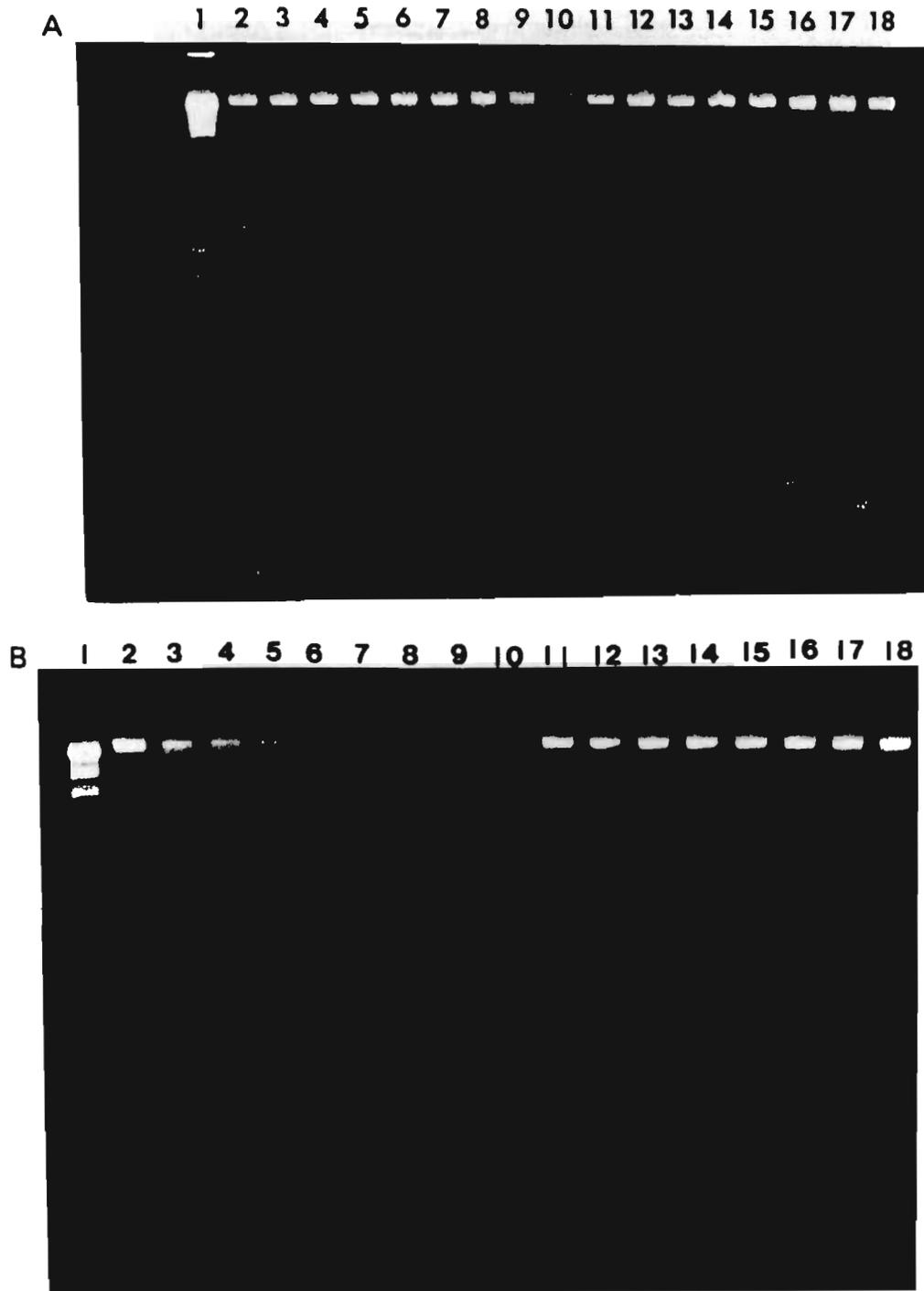


Fig. 4 Time course of DNA degradation by cassava (A) and soybean (B) tissue extracts visualized by U.V. fluorography after electrophoresis on a 1.2% agarose gel. Lane 1, lambda DNA digested with *Hind* III as markers; lane 2 and 11 negative controls, phage DNA without crude tissue extract incubated for 60 and 180 min, respectively; lanes 3-10 contain 1 µg of phage DNA incubated with 0.2 µg of root extract for 1, 2, 5, 10, 15, 20, 30 and 60 min, respectively. Lanes 12-18 contain 1 µg phage DNA incubated with 4 µg of leaf extract for 15, 30, 60, 90, 120, 150 and 180 min, respectively.

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this possibility bacterial  $\beta$ -glucuronidase or firefly luciferase was incubated with various amounts of root and leaf extracts and the relative activity was determined. As shown in Fig. 3, root extracts inhibited  $\beta$ -glucuronidase activity in a concentration-dependent manner. The addition of 20  $\mu$ g protein of cassava root extract (equivalent to 13% of the total assay volume) inhibited GUS activity by 34%. Similarly, an equivalent volume of soybean root extract inhibited GUS activity by 23%. Leaf extracts of both plants, however, were less effective in inhibiting GUS activity. Expressed on a protein basis, 20  $\mu$ g of cassava root protein extract was 5-fold more effective in inhibiting  $\beta$ -glucuronidase activity than was an equivalent amount of cassava leaf protein extract. Since the inhibition of GUS activity was rapid ( $< 1$  min) and did not increase with extended preincubation ( $\leq 60$  min) of GUS with tissue extracts it is unlikely that the inhibition of GUS activity was due to proteolytic activity. Furthermore, the addition of PMSF, a serine type proteinase inhibitor, to tissue extracts did not alter GUS activity (data not shown). In contrast to their effects on GUS activity, neither cassava leaf or root extracts inhibited luciferase activity (data not shown). On the contrary, there was a slight enhancement of luciferase activity from added cassava root and/or leaf extracts. These results indicate that the relative difference in GUS versus luciferase expression may in part result from inhibition of GUS activity in roots. These results, however, do not account for the low efficiency of transient transformation of roots when using the luciferase reporter gene.

Another means by which the efficiency of transient gene expression can be reduced is by degradation of the introduced DNA by endogenous nuclease activity [23]. In order to determine whether nuclease activity was present in cassava and soybean tissues, crude tissue extracts were incubated with lambda DNA for various time intervals followed by determination of the DNA integrity by gel electrophoresis (Fig. 4). In the presence of cassava root extract (0.2  $\mu$ g protein), lambda DNA was substantially degraded in as little as 10 min. In contrast, there was little evidence of DNA degradation in the presence of 20-fold higher levels of leaf protein (4  $\mu$ g) even after 2 h of incubation (Fig. 4A). Similar to cassava, soybean root extracts had high levels of DNase activity whereas leaf extracts did not (Fig. 4B). The level of DNase activity in cassava tissues was then determined by quantification of the amount of TCA precipitable  $^{32}$ P-labeled lambda phage DNA following incubation with crude tissue extracts. Reduced levels of precipitated DNA would indicate the generation of free, non-precipitable nucleotides via the activity of DNase(s). As indicated in Table 3, there was a 26% and 8% reduction in the level of precipitated labeled phage DNA when incubated with cassava root and leaf crude tissue extract (0.3  $\mu$ g protein), respectively. This represents a 4-fold higher apparent level of DNase activity in roots than leaves. The high level of DNase activity in roots was even more apparent when we used reduced amounts of tissue extracts (0.15  $\mu$ g of protein, note: lesser amounts of tissue extract did not alter the ratio of root/leaf degraded

Table 3  
Comparative analyses of the DNase activity of cassava root and leaf protein extracts

Tissue	Tissue extract ( $\mu$ g protein)	Precipitable DNA (counts/min)	Intact DNA (%)	Degraded DNA (%)	Degraded DNA root/leaf ratio
Control	0	64 329	100	0	
Root	0.3	47 840	74	26	
Leaf	0.3	59 444	92	8	3.25
Root	0.15	52 167	81	19	
Leaf	0.15	61 476	96	4	4.75

The activity is expressed as the percentage of intact DNA following incubation with or without crude tissue extracts. See Materials and methods for details. Each value corresponds to the average of 2 assays from three different tissue extractions.

DNA, Table 3) in the assay. While the loss in TCA precipitable counts indicates that there is higher DNase activity in root than in leaf tissue, this assay may underestimate the effect of root DNase activity on the integrity of introduced DNA since only the loss of free nucleotides and not precipitable oligonucleotide fragments would be detected. It is apparent from Fig. 4A that the DNase activity in cassava roots can cause substantial (sequence independent) damage to high molecular weight DNA (note: similar effects were observed when using pBI221 plasmid DNA as a substrate). At present, the cellular location of the DNase activity is not known, however, preliminary studies indicate that the pH optimum for this activity is between 6.0-6.5, suggesting that it may not be cytoplasmic.

In summary, it is apparent that efficiency of transient transformation is substantially reduced in roots relative to leaf tissues. The fact that neither a broad tissue range promoter such as the 35S CaMV or the root specific promoter glutamine synthetase were expressed at high levels in either cassava or soybean roots suggests that factors other than promoter specificity may limit transient and subsequent stable transformation efficiency. The reduced expression of GUS in transformed roots can in part be attributed to GUS enzyme inhibitors. GUS inhibitors are, however, unlikely to account for the reduced levels of transformation, since luciferase expression was also substantially reduced in cassava root tissues relative to leaf tissue. Therefore, it is likely that other factors reduce the efficiency of transient transformation in cassava roots. The presence of high levels of DNase activity in roots and the absence of significant DNase activity in leaves most likely accounts for the reduced transient transformation efficiency of cassava and soybean roots. Transforming DNA which is delivered by microprojectiles passes through a variety of cell compartments prior to arrival in the nucleus. Root DNase(s) could presumably degrade or damage the introduced DNA thereby reducing transformation efficiency. Therefore, in order to identify root-specific promoters in cassava it will be necessary to use other experimental systems including analysis of reporter gene expression in

stable transformed plants or in transiently-transformed protoplasts. Last of all, we note that the high levels of DNase activity in roots may serve to protect the plant from viral and bacterial pathogens.

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## 6. References

- 1 J.H. Cock. Cassava: a basic energy source in the tropics. *Science*, 218 (1982) 755-762.
- 2 T. Eksittikul and M. Chulavatnatol, Characterization of cyanogenic B-glucosidase from cassava (*Manihot esculenta* Crantz). *Arch. Biochem. Biophys.*, 266 (1988) 263-269.
- 3 C. Bagalopalan, G. Padmaja, S. Nanda and S.Morthy. Cassava nutrition and toxicity, in: *Cassava in Food, Feed and Industry*. CRC Press, Boca Raton, FL 1988, pp. 13-36.
- 4 T. Tylleskar, M. Banea, N. Bikangi, R. Cooke, N. Poulter and H. Rosling, Cassava cyanogens and konzo, an upper motoneuron disease found in Africa. *Lancet*, 329 (1992) 208-211.
- 5 O. Mkpog, H. Yan, G. Chism and R.T. Sayre, Purification, characterization, and localization of linamarase in cassava. *Plant Physiol.*, 93 (1990) 176-181.
- 6 P.N. Benfey, L. Ren and N.-H. Chua, The CaMV 35S enhancer contains at least two domains which can confer different developmental and tissue-specific expression patterns. *EMBO J.*, 8 (1989) 2195-2202.
- 7 P.N. Benfey and N.-H. Chua, The cauliflower mosaic virus 35S promoter: combinatorial regulation of transcription in plants. *Science*, 250 (1990) 959-966.
- 8 C. Franche, D. Bogusz, C. Schopke, C. Fauquet and R. Beachy, Transient gene expression in cassava using high-velocity microprojectiles. *Plant Mol. Biol.*, 17 (1991) 493-498.
- 9 R.A. Jefferson, T.A. Kavanagh and M.W. Bevan, GUS fusions: B-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.*, 6 (1987) 3901-3907.
- 10 W.M. Roca, Cassava, in: W.R. Sharp, D.A. Evans, P.V. Ammirato, Y. Yamada (Eds.), *Handbook of plant cell culture*, Vol. 2 Crop Species. MacMillan Publishing, New York, 1984, pp. 269-301.
- 11 T. Murashige and F. Skoog, A revised medium for rapid

- growth and bioassays with tobacco tissue cultures. *Physiol. Plant.*, 15 (1962) 473-497.
- 12 M. Guo-Hua, B. Hirel, M.C. Marsolier, R.W. Ridge and D.P. Verma, Ammonia-regulated expression of soybean gene encoding cytosolic glutamine synthetase in transgenic *Lotus corniculatus*. *Plant Cell*, 3 (1991) 11-22.
  - 13 D.W. Ow, K.V. Wood, M. DeLuca, J.R. de Wet, D.R. Helinski and S.H. Howell, Transient and stable expression of the firefly luciferase gene in plant cells and transgenic plants. *Science*, 234 (1986) 856-859.
  - 14 J.J. Finer and M.D. McMullen, Transformation of cotton (*Gossypium hirsutum* L.) via particle bombardment. *Plant Cell Rep.*, 8 (1990) 586-589.
  - 15 J.J. Finer, P. Vain, M.W. Jones and M.D. McMullen, Development of a particle inflow gun for delivery of DNA to plant cells. *Plant Cell Rep.*, (1992) in press.
  - 16 R.A. Jefferson, Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Mol. Biol. Rep.*, 5 (1987) 387-405.
  - 17 M. Bradford, A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, 72 (1976) 248-254.
  - 18 T. Maniatis, E.F. Fritsch and J. Sambrook, *Molecular Cloning, A laboratory manual*. Cold Spring Harbor Laboratory, New York, 1982.
  - 19 Y.-C. Wang, T.M. Klein, M. Fromm, J. Cao, J.C. Sanford and R. Wu, Transient expression of foreign genes in rice, wheat and soybean cells following particle bombardment. *Plant Mol. Biol.*, 11 (1988) 433-439.
  - 20 M.J. Battraw and T.C. Hall, Histochemical analysis of CaMV 35S promoter-B-glucuronidase gene expression in transgenic rice plants. *Plant Mol. Biol.*, 15 (1990) 527-538.
  - 21 S. Subramani and M. DeLuca, Applications of the firefly luciferase as a reporter gene. in: J.K. Setlow (Ed.), *Genetic engineering: Principles and Methods*. Plenum Press, New York, 10, 1988, pp. 75-89.
  - 22 A.J. Millar, S.R. Short, N.-H. Chua and S.A. Kay, A novel circadian phenotype based on firefly luciferase expression in transgenic plants. *Plant Cell*, 4 (1992) 1075-1087.
  - 23 B. Hughes, F. White and M. Smith, Fate of bacterial plasmid DNA during uptake by barley and tobacco protoplasts: II protection by poly-L-ornithine. *Plant Sci. Lett.*, 14 (1979) 303-360.