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CONTENTS

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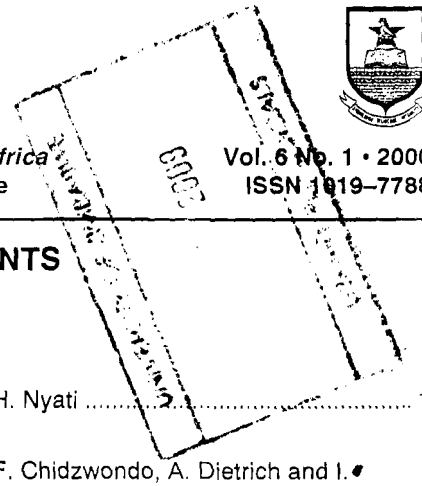

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The *Mae* I assay for scoring atrazine resistance is codon-usage dependent in legumes

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Nine different cowpea varieties were grown and total deoxyribonucleic acid (DNA) isolated from the young leaves. This DNA was used to amplify a 291 base pair fragment that contained codon 264 of the *psb A* gene. The primers used were designed based on the soybean *psb A* gene sequence. The 291 base pair fragment was digested with the restriction enzyme *Mae* I to assay for sensitivity/resistance to atrazine using a published method. When *Mae* I did not cut the 291 base fragment, the whole gene was amplified by the polymerase chain reaction, cloned into pBSK+II (Stratagene, CA, USA) and sequenced around codon 264. Codons 263 and 264 were found to be GCA and AGT respectively. UWGCG and BLAST searches of the *psb A* gene sequences in the databases confirmed that *Mae* I has a recognition site that overlaps with codons 263 and 264 in some legumes but not in others. The *Mae* I assay used by Cheung and co-workers is therefore not universal but depends on codon usage in legumes.

Keywords: *psb A* gene, atrazine, codon usage, DI protein, plastoquinone

Introduction

The herbicide atrazine has a wide spectrum of activity and is used for selective pre-emergence and early post-emergence control of annual broadleaf and grassy weeds (Holt *et al.*, 1993). In Zimbabwe atrazine is used widely, especially on maize fields. The maize lines grown in this country are naturally resistant to atrazine due to the presence of two glutathione-S-transferase isoforms that detoxify atrazine (Mbaya, Shell Agrichemicals, personal communication). Atrazine is, however, slowly metabolised to inactive forms in the soil (Hassall, 1990). Consequently, residual effects limit the farmers' choice of crop rotation as some crops are sensitive to atrazine. These limitations could be overcome if atrazine-resistant crops were available.

Atrazine's mode of action is through the destruction of photosynthetic tissue (Steinback *et al.*, 1981; Erickson *et al.*, 1985). The triazine acts by binding to the DI

protein, a 32 kDa thylakoid membrane polypeptide of photosystem II (PSII) which is encoded by the highly conserved chloroplast *psb A* gene (Erickson *et al.*, 1985; Hanley-Bowdoin and Chua, 1988; Ohad and Hirshberg, 1992). The DI protein is the apoprotein of quinone co-factor B (Q_B), the second stable electron acceptor of PSII. When atrazine binds to the DI protein, it displaces the plastoquinone Q_B from its binding site thereby blocking electron transport in PSII (Rochaix and Erickson, 1988; Ohad and Hirshberg, 1992). The electrons formed by photolysis of water are consequently shunted into the chloroplast stroma where they form highly reactive free radicals such as superoxides. These unstable free radicals oxidise and destroy chlorophylls, carotenoids and other pigments in the leaves leading to chlorosis, necrosis and death of leaves (Hassall, 1990).

Plants that are resistant to atrazine are still able to synthesize the DI protein but binding of azido-[^{14}C]-atrazine to this protein is greatly reduced (Steinback *et al.*, 1981). This suggests that there is a subtle change in the DI protein from resistant plants that results in a decrease in herbicide binding. The *psb A* gene, which encodes the DI protein, has been cloned and the nucleotide sequence determined from a number of higher plants such as *Amaranthus hybridus* (Hirshberg and McIntosh, 1983) and *Solanum nigrum* (Goloubinoff *et al.*, 1984).

A number of mutant *psb A* genes have also been isolated and sequenced. This has made it possible to identify amino acid substitutions that confer atrazine resistance. In the higher plants a substitution of serine residue at codon 264 with a glycine residue results in resistance to atrazine (Steinback *et al.*, 1981; Hirshberg and McIntosh, 1983; Goloubinoff *et al.*, 1984).

Cheung and co-workers (1993) developed an assay to distinguish atrazine-sensitive plant varieties from resistant varieties and tested the assay in *Brassica napus, Chenopodium spp* and *Amaranthus spp.* The method involves polymerase chain reaction (PCR) amplification of the specific *psb A* region containing codon 264 coupled with *Mae* I digestion. The assay is based on the fact that the *Mae* I restriction site C\TAG that overlaps with codon 264 is abolished by a point mutation in resistant plants. Resistant plants therefore give one fragment instead of two.

The long-term objective of this project was to produce atrazine resistant cowpeas. It was therefore necessary to assay several cowpea varieties for sensitivity/resistance to atrazine to check for the possibility of there being any that are resistant.

Materials and Methods

Cowpea seeds were obtained from the Department of Research and Specialist Services, Ministry of Lands and Agriculture, Zimbabwe.

Assaying for sensitivity/resistance of cowpeas to atrazine

Seeds from nine different varieties of cowpeas namely CB5, 475/89 (local variety), IT82D-789, IT82D-875, IT82D-889, IT83S-872, IT84D-666, TVx-1948-OIF and VITA4 were planted. DNA was isolated from the young tender leaves using the method used by Cheung *et al.*, (1993).

Primers, designated 8729 and 8695, designed from the published soybean *psb* A gene sequence (EMBL Accession No. X00152) were used in PCR to amplify the fragment that contained codon 264. This 291 bp fragment was digested with the restriction enzyme *Mae* I and analysed by resolution on a 1 percent agarose gel (Cheung *et al.*, 1993).

Cloning of the cowpea psb A gene

The coding region of the *psb* A gene was amplified by PCR using primers designated 9396 and 9394. These primers, also designed from the soybean *psb* A gene sequence, had *Eco* R1 recognition sites added to the ends. The PCR-amplified fragments were purified by electroelution, digested with *Eco* R1 and cloned into the *Eco* R1 site in the polylinker of the plasmid pBSK+*II* (Stratagene, CA, USA). The cloned fragments were sequenced around codon 264 using the method of Sanger.

Computer-aided sequence alignments and restriction enzymes sites of the psb A sequences

Sequence alignments were run with the BLAST 2.0 software of the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast/blast.cgi) or with the UWGCG 8.1 Sequence Analysis Software Package from the University of Wisconsin Genetics Computer Group (hptt//www.gcg.com). Restriction enzyme mapping was carried out using the UWGCG 8.1 or the DNA Strider 1.2 (C. Marck, marck@jonas.saclay.cea.fr) software.

Results

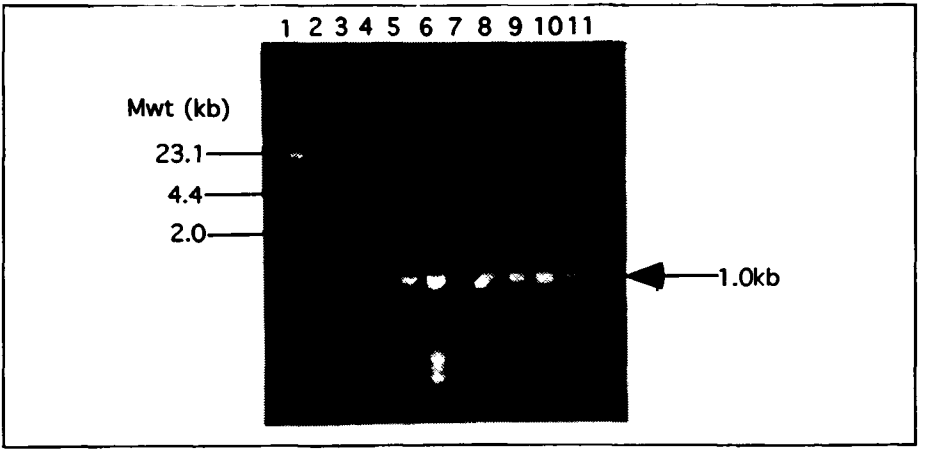

Figure 1: Resolution of the PCR-amplified coding region of the *psb A* gene from nine cowpea varieties. Lane 1 shows the lambda *Hind* III Mw size markers. Lane 2 shows the negative control (no template DNA). Lanes 3 to 11 show the varieties CB5, 475/89 (local variety), IT82D-789, IT82D-875, IT82D-889, IT83S-872, IT84D-666, TVx-1948-OIF and VITA 4 respectively.

Amplification of the cowpea psb A gene by PCR

The coding region of the *psb A* gene from all the nine different varieties was successfully amplified by PCR. Figure 1 shows the resolution of the amplified coding region of the psb A gene on a one percent agarose gel.

The Mae I assay

The 291 base pair fragment of the *psb A* gene containing codon 264 was amplified from all nine cowpea varieties. When these fragments were digested with *Mae* I, none of them was cut as is shown in Figure 2.

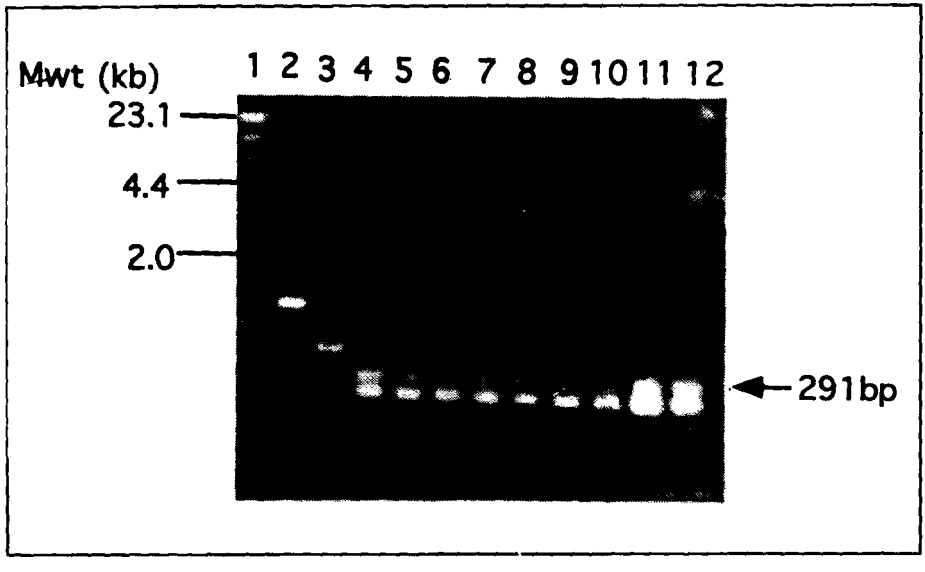

Figure 2: *Mae* I restriction pattern of the 291 base pair PCR-amplified *psb A* fragment containing codon 264 from nine cowpea varieties. Resolution is on a 1 percent agarose gel. Lane 1 shows the lambda Hind III Mw size markers. Lanes 2 and 3 show the undigested and digested *psb A* gene respectively. Lanes 4 to 12 show the varieties CB5, 475/89 (local variety), IT82D-789, IT82D-875, IT82D-889, IT83S-872, IT84D-666, TVx-1948-OIF and VITA 4 respectively.

5'GTAGCTGCTCATGGTTATTTTGGCCGATTAATCTTCCAATATGCAAGTTTC
AACAATTCTCGTTCTTTACATTTCTTCCTAGCTGCTTGGCCTGTAGTAGGTA
TTTGGTTTACCGCTTTAGGTATCAGCACTATGGCTTTCAACTTAAATGGTTTT
AATTTCAACCAATCCGTAGTTGAT 3'

Figure 3: Sequence around codon 264 of the cowpea *psb A* gene. Codons 263 and 264 are shown in bold.

Mae I X80932	ctag gattaatcttccaatatg**caag**tttcaacaattctcgttctttac	Cowpea (*Vigna unguiculata*)
(A)		
X04973	----g---------**t**-----------c----------	Alfalfa (*Medicago sativa*)
X17694	----g---------**t**---------------c------	Broad bean (*Vicia faba*)
M11005	----g---------**t**---------------c------	Pea (*Pisum sativum*)
X86563	---------------**t**--------------------	Maize (*Zea mays*)
X07521	---------------**t**---------c----------	Barley (*Hordeum vulgare*)
X13327	---------------**t**---------c----------	Rye (*Secale cereale*)
M36191	---------------**t**-----t----c----------	Rice (*Oryza sativa*)
(B)		
X00152	----g---------**------**--------------------	Soybean (*Glycine max*)
X68048	----g---a-----**------**--------------------	Common bean (*Phaseolus vulgaris*)
M36720	----g---------**tg**--------------------	Rape-seed (*Brassica napus*)
AB020622	----------c-----c----t----c----------	Liver-wort (*Dumortiera hirsuta*)
U25659	----g---------**tg**---------c------g----	Black nightshade (Solanum nigrum)
X08017	----g---------**t-a**---------c------g-----	Curled- leaved tobacco (*Nicotiana plumbaginifolia*)
AB025940	----g---------**t-c**---------c------g----	Tobacco(*Nicotiana tabacum*)
X52758	------t---------**------**-t--t----a----a----	Red alga (*Cyanidium caldarium*)
X62800	-t----------c--**ttc**---------c--------c-	Green alga (*Chlorella ellipsoidea*)
X79223	-t----------c--**ttca**---------c------g----	Yellow-green alga (*Bumilleriopsis filiformis*)
X02350	-tc----------c--**ttc**----c--------a----	Green alga(*Chlamydomonas reinhardtii*)
Z49201	-c----------**--g**----t--t--cagc--cag-c-t-	Blue-green alga (*Prochlorococcus marinus*)

Figure 4: Alignment of the sequence around codon 264 in the cowpea *psb A* gene with the corresponding sequence in the *psb A* gene of a number of other species. Only the nucleotides which differ from the cowpea sequence are indicated. Codons 263 and 264 are underlined. The *Mae* I recognition motif is given at the top. The *psb A* genes in panel (A) possess the *Mae* I site spanning over codons 263 and 264, the genes in panel (B) do not. The nucleotide positions concerned by the presence or absence of this site are in bold in all sequences. The database accession numbers of the genes presented are given on the left side.

Nucleotide sequence around codon 264 of the cowpea psb A gene

The sequence around codon 264 was identical in all the nine cowpea varieties sequenced (Figure 3). In all cases, alanine 263 is specified by a GCA codon instead of the GCT codon required for the *Mae* I assay, whereas codon 264 (AGT) still specifies serine. As a consequence, the absence of the *Mae* I site is not associated with resistance to atrazine in cowpea.

Codons 263 and 264 in the psb A genes from other species

The *psb A* gene sequence of more than a hundred different species can currently be found in the databases. The vast majority of these sequences do contain the *Mae* I site spanning over codons 263 and 264, including major crops like maize, barley, rye or rice and the legumes alfalfa, broad bean and pea (Figure 4A). However, in the *psb A* genes of the legumes soybean and common bean, the sequence of codons 263 and 264 is the same as in the cowpea *psb A* gene and therefore it lacks the *Mae* I recognition motif, although there is no change in amino acid 264 (Figure 4B). The rapeseed and black nightshade genes contain the A to G switch at the first position of codon 264 associated with both atrazine resistance and disappearance of the *Mae* I site, whereas the liverwort and tobacco *psb A* have amino acid conservative changes leading to the loss of the *Mae* I motif. Strikingly, the latter is also true for all types of algae (Figure 4B).

Discussion

The nine different cowpea varieties were assayed for sensitivity/resistance using the method developed by Cheung and co-workers (1993). In the three plant species (*Amarathus, Brassica* and *Chenopodium*) that they assayed, the nucleotide sequence for alanine codon 263 is GCT which forms part of the *Mae* I recognition sequence CTAG. A computer-aided restriction sites search of the soybean *psb* A gene sequence (EMBL Accession No. X00152) showed that *Mae* I does not have a recognition site within the amplified fragment and hence the assay does not work for soybean. Digestion of the cowpea 291 bp fragment with *Mae* I showed that there is no recognition site for this restriction enzyme in this region of the cowpea *psb A* gene (Figure 2). This observation was confirmed by sequencing (Figure 3).

The fact that none of the fragments from the nine cowpea varieties was cut by *Mae* I led to three possible conclusions. The recognition site could have been absent because alanine codon 263 is GCA, just like in soybean, or because of a point mutation at the first position of codon 264 from an A to a G, (serine to glycine), or both. Whichever way the assay could not be used to distinguish sensitive varieties from resistant ones. In order to determine this, the gene from all the nine varieties was cloned and sequenced. The sequence from codon 263 to 265 is GCAAGTTTC (Figure 3) just like in soybean. From the sequence codon 264 codes for a serine residue, indicating that all the cowpea varieties assayed are sensitive to atrazine (Steinback *et al.*, 1981; Hirshberg and McIntosh, 1983; Goloubinoff *et al.*, 1984).

A search of the *psb A* gene sequences from other legumes showed that, whereas GCT is by far the preferred codon for alanine 263 of the DI protein in higher plants, legumes split by half into species using GCT (alfalfa, broad bean, pea) and species using GCA (common bean, cowpea, soybean).

In the latter case, *Mae* I does not have a recognition site that overlaps with codon 264. Hence, the assay proposed by Cheung and co-workers (1993) for testing atrazine resistance in plants, although widely applicable, is dependent on codon usage in legumes.

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REFERENCES

CHEUNG, W. Y., COTE, J. C., BENOIT, D. L. AND LANDRY, B. S. 1993 A rapid assay for chloroplast-encoded triazine resistance in higher plants. *Plant MolecularBiology Reporter* **11**: 142–155.

ERICKSON, J. M., RAHIRE, M. AND ROCHAIX, J. D. 1985 Herbicide resistance and cross resistance: changes at three distinct sites in the herbicide-binding protein. *Science* **228**: 204–207.

GOLOUBINOFF, P., EDELMAN, M. AND HALLICK, R. B. 1984 Chloroplast-coded atrazine resistance in *Solanum nigrum: psb A* loci from susceptible and resistant biotypes are isogenic except for a single codon change. *Nucleic Acids Research* **12**: 9489–9496.

HANLEY-BOWDON, L. AND CHUA, N. 1988 Transcription of the wheat chloroplast gene that encodes the 32 kd polypeptide. *Plant Molecular Biology Reporter* **10**: 303–310.

HASSALL, K. A. 1990 *The biochemistry and uses of pesticides: structure, metabolism, mode of action and uses in crop protection.* Macmillan ELBS, Hong Kong, 441–454.

HIRSCHBERG, J. AND MCINTOSH, L. 1983 Molecular basis of herbicide resistance in *Amarathus hybridus. Science* **222**: 1346–1349.

HOLT, J. S. AND HOLTUM, J. A. M. 1993 Mechanisms and agronomic aspects of herbicides resistance. *Annual Review of Plant Physiology and Plant Molecular Biology* **44**: 203–229.

OHAD, N. AND HIRSHBERG, J. 1992 Mutations in the D1 subunit of photosystem II distinguish between quinone and herbicide binding sites. *The Plant Cell* **4**: 273–282

ROCHAIX, J. D. AND ERICKSON, J. 1988 Function and assembly of photosystem II: genetic and molecular analysis. *Trends in Biochemical Sciences* **13:**, 56–59.

STEINBERG, K. E., MCINTOSH, L., BOGORAD, L., AND ARNTZEN C. J. 1981 Identification of the triazine receptor protein as a chloroplast gene product. *Proceedings in National Academy of Science* USA **78**: 7463–7467.

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