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Review paper

Synthetic *bxn* Gene Utilization in the Resistance of Crops to the Herbicide Bromoxynil – A Review

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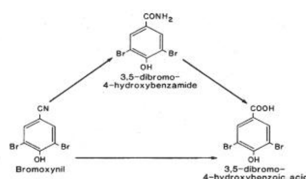
Graphical abstract



Abstract

Modern biotechnological systems refer to the application of biological methods for the production of new substances and manipulation of genetic materials of living organisms for the benefits of human future and commercial applications. Generation of herbicide resistant crops is a very important aspect of biotechnology utilization in plant genetic engineering by using genetic materials of another plant species or organisms to generate herbicide resistant transgenic crops. Nitrile group of herbicides is a large group of chemicals mostly inhibit photosynthesis in weeds and causes many loses in agricultural crops. The bromoxynil-specific nitrilase (*bxn*) gene was used widely in the production of crops resistant to the herbicide Bromoxynil. The gene was originally isolated from the soil bacterium *Klebsiella pneumoniae* subsp. *ozaenae* and was successfully transformed into plant or appropriate crops of commercially interest. The encoded protein catabolizes 3,5-dibromo-4-hydroxybenzonitrile (Bromoxynil) to non-phytotoxic 3,5-dibromo-4-hydroxybenzoic acid. This review described the application of synthetic and codon-optimized *bxn* gene for the efficient transformation into plant resistance to Bromoxynil.

Keywords: *Bxn* gene; bromoxynil; synthetic gene; plant transformation; plant biotechnology

Abstrak

Sistem bioteknologi moden merujuk kepada permohonan kaedah biologi untuk pengeluaran bahan-bahan baru dan manipulasi bahan-bahan genetik organisma hidup bagi manfaat masa depan manusia dan aplikasi komersial. Generasi tanaman tahan herbisid adalah aspek yang sangat penting penggunaan bioteknologi dalam bidang kejuruteraan genetik tumbuhan dengan menggunakan bahan-bahan genetik lain spesies tumbuhan atau organisma untuk menjana herbisid tahan transgenik tanaman. Kumpulan nitril racun rumpai adalah satu kumpulan besar bahan kimia kebanyakannya menghalang fotosintesis dalam rumpai dan menyebabkan banyak kehilangan dalam tanaman pertanian. Bromoxynil-gen tertentu nitrilase (bxn) telah digunakan secara meluas dalam pengeluaran tanaman tahan kepada Bromoxynil racun herba tersebut. Gen pada asalnya diasingkan daripada tanah bakteria Klebsiella pneumoniae subsp. ozaenae dan telah berjaya berubah menjadi tumbuh-tumbuhan atau tanaman yang sesuai kepentingan komersial. Protein dikodkan catabolizes 3,5-dibromo-4-hydroxybenzonitrile (Bromoxynil) bukan phytotoxic asid 3,5-dibromo-4-hydroxybenzoic. Kajian ini menyifatkan permohonan gen bxn sintetik dan codon-dioptimumkan bagi transformasi cekap ke dalam loji rintangan untuk Bromoxynil.

Kata kunci: Bxn gen; bromoxynil; sintetik gen; transformasi tumbuhan; bioteknologi tumbuh-tumbuhan

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■1.0 INTRODUCTION

Modern biotechnology refers to the technical applications of biological systems for the production of substances that involves manipulation of living organisms for the benefit of human for commercial purposes (Soetan, 2008). Biotechnology employs useful and simple procedures to identify, isolate, purify and study the regulation of genes and their applications. It also empowers the transfer of genes from one organism to another (Osuntoki, 2005). Plant biotechnology is expected to have immediate practical applications in the field of weed management by production of herbicide-tolerant transgenic crops which many consider to be more sustainable. Herbicides have different mode of action upon plants such as growth regulators, seedling growth inhibitors, photosynthesis inhibitors, amino acid synthesis inhibitors, lipid synthesis inhibitors, cell membrane disrupter and pigment inhibitors.

The ability of naturally occurring soil microorganisms to degrade a wide variety of organic compounds has been reviewed extensively (Frantz and Chakrabarty 1986; Gibson, 1984; Wollaston *et al*., 1992). Current interest is to develop bromoxynil-specific nitrilase (*bxn*) gene (Stalker and Mcbride 1987) able to hydrolyzes bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) the active material of herbicide to the non-phytotoxic (3,5-dibromo-4-hydroxybenzoic acid) using synthetic gene. Most of the genes are plasmid encoded. However, the drawback of this approach is that

often the target organism does not have the same 'codon-usage' profile as the organism that the gene was isolated from. It has been shown that in most organisms there is a preference for some codons coding for a specific amino acids, while other codons, used for the same amino acid are used less frequently. Since this 'codon-usage' is organism-specific, a direct transfer of a gene from one organism to another might lead to sub-optimal expression levels of the encoded proteins.

To increase the level of transgenic protein levels, there has been much interest in the use of "codon-optimized" synthetic genes for transgenic plant production (Perlak *et al*., 1990; Adang *et al*., 1993; Koziel *et al*., 1993). In this case, the original gene is modified in such a way that the codon-usage profile matches the profile of the host organism.

In this review we assess the application of a synthetic and codon-optimized *bxn* gene in crops to confer resistance to the herbicide Bromoxynil.

1.1 Bacteria that Contain *bxn* Gene in Their Genome

One study on the presence of nitrile-degrading activity can be obtained from bacteria and actinomycetes isolated from soil and deep-sea samples of wide-ranging geographical origins. For example, Bromoxynil-metabolising gram-negative bacteria were detected in soil samples from Argentina and Namibia (Brandao *et al*., 2002). The genetic basis for the ability of soil bacteria to degrade organic compounds resides on naturally occurring plasmid elements (Stalker and Mcbride 1987). For instance, *Desulfitobacterium chlororespirans* have been shown to undergo metabolic reductive dehalogenation by the microorganism (Cupples *et al*., 2005). Previously, a soil bacterium, *Klebsiella ozaenae*, was isolated. This bacterium utilizes bromoxynil as a sole source of nitrogen and that encodes a nitrilase that rapidly converts bromoxynil to its metabolite 3, 5-dibromo-4-hydroxybenzoic acid (McBride *et al*., 1986). The *bxn* gene used for genetic improvement of crops has been cloned from an isolate of the bacterium *Klebsiella pneumonia* subsp. *ozaenae. Klebsiella pneumonia* subsp. *ozaenae* was found in bromoxynil contaminated soil. This isolate was capable of growing on bromoxynil-containing media and utilizing the ammonia released from converted bromoxynil as its sole source of nitrogen (McBride *et al*., 1986; Stalker *et al*., 1988). So far, there is no new isolated gene for application in genetic transformation of crops resistant to herbicide bromoxynil.

1.2 Isolation and Properties of *bxn* Gene

The *bxn* gene from *Klebsiella ozaenae* is constitutively expressed in *Escherichia coli* after direct transfer of a 82-kilobase (kb) plasmid. The gene comprises 1212 bp nucleic acid and the coding sequence is from position 66 (ATG) to position 1115 (TAA) (Stalker *et al*., 1988; NCBI Accession number J03196). The gene contains a single open reading frame encoding a polypeptide of 349 amino acids in length.

1.3 Assay of the *bxn* Protein (Nitrilase)

There are several procedures have been introduced in order to assay the *bxn* encoded protein. All enzyme assays measure either the consumption of substrate or production of product over time. Bromoxynil degradation by nitrilase activity can be measured by determining the release of ammonia from the nitrile moiety of bromoxynil (Harper, 1977). Ammonium chloride concentrations ranging from 0 to 1.5 L/mol per assay was used to construct a standard curve. The specific activity can be measured as micromoles of NH_3 released per minute per milligram of protein. Some of the assays include:

a) Spectrophotometric assays, is to follow the course of the reaction by measuring a change in how much light the assay solution absorbs. If this light is in the visible region it can actually see a change in the color of the assay or known as colorimetric assays.

b) Calorimetry assay is the measurement of the heat released or absorbed by chemical reactions. These assays are very general, since many reactions involve some change in heat and with use of a microcalorimeter, where not much enzyme or substrate is required (Todd and Gomez, 2001).

c) Chromatographic assays measure product formation by separating the reaction mixture into its components by chromatography. This is usually done by high-performance liquid chromatography (HPLC), but can also use the simpler technique of thin layer chromatography. This approach requires a lot of material. Its sensitivity can be increased by labelling the substrates/products with a radioactive or fluorescent tag. Assay sensitivity has also been increased by switching protocols to improved chromatographic instruments (e.g. ultra-high pressure liquid chromatography) that operate at pump pressure (Churchwella, *et al*., 2005). Other enzyme assay methods commonly used are: Fluorometric, Chemiluminescent, Light Scattering, Microscale Thermophoresis and Radiometric and will not be discussed in this review.

1.4 Degradation Pathway and Mode of Action in Bromoxynil

The *bxn* nitrilase converts the 3,5-dibromo-4-hydroxybenzonitrile to its metabolite 3,5-dibromo-4-hydroxybenzoic acid which is at least 100-fold less toxic (Figure 1).

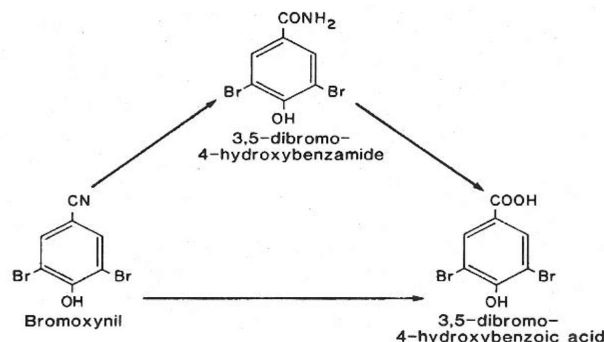


Figure 1 Hydrolyzing bromoxynil to its non-toxic benzoic acid by nitrilase enzyme in plants (adopted from Mc Bride *et al*., 1986)

Bromoxynil is a nitrile herbicide that blocks photosynthesis, the food production process in the plants. The primary mode of action for bromoxynil (3,5-dibromo-4-hyroxybenzonitrile) is to inhibit photosynthesis by binding to the Photosystem II complex of chloroplast membranes and blocking electron transport (Ahrens, 1994). Symptoms of injury are leaf margin burn and interveinal chlorosis. Normally, the older leaves are affected first. The bromoxynil herbicide profile is shown in Table 1.

Table 1 The basic information of bromoxynil herbicide (Sigma-aldrich)

Chemical Name	3,5-Dibromo-4-hydroxybenzonitrile
Trade Name(s)	Bronate, Buctril, Brominal
Molecular Formula	$C_7 H_3 Br_2 NO$
Molecular Weight	276.9
Physical State	Colorless solid (pure compound)
Odor	Odorless when pure
Melting Point	194-195 °C
Solubility	130 mg/l water at 25 °C

1.5 Application of the *bxn* Gene In Herbicide-Resistant Plants

In USA back in 2003, 81% of the soybean crop, 59% of the upland cotton and 15% of the maize were herbicide tolerant (Benbrook, 2003). Initial study was to transfer the *bxn* gene and express its bromoxynil-specific nitrilase in leaves of transgenic tobacco plants conferred resistance to high levels of a commercial formulation of bromoxynil (Stalker *et al*., 1988). A similar approach in potato plants showed about 70-fold higher resistance to bromoxynil in transgenic potato plants as compared to untransformed potato plants (Eberlein *et al*., 1998). Bromoxynil resistance transgenic tomato, commercial oil seed rape (Canola) and cotton has created by Calgene, USA. Bromoxynil-Resistant Common Groundsel (*Senecio vulgaris* L.) has been reported by Mallory-Smith, 1998 and in subterranean clover (Dear *et al*., 2003).

■2.0 SYNTHETIC GENES

2.1 Advantages of Using Synthetic Genes

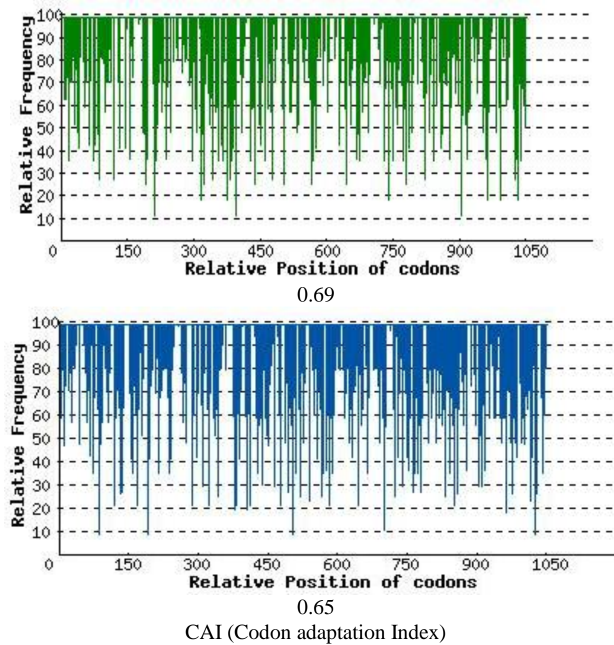
Synthetic genes are to provide an immediate and easy path from sequence databases and sequence manipulation software to physical DNA, enabling research that would not be possible or practical relying on natural DNA sequences. Traditional cloning is limited by the existing natural DNA sequence of the gene, which encodes the codon bias, cis-regulatory elements, and restriction sites of the organism from where it was derived. Gene synthesis has became an important tool in many fields of recombinant DNA technology including heterologous gene expression, vaccine development, gene therapy and molecular engineering. Codon optimization can result in a host-specific codon usage profile, thus leading to enhance expression levels of the encoded proteins (Welch *et al*., 2009). Furthermore, codon optimization can also result in secondary structure modification of the transcribed mRNA.

The increased of protein levels can be achieved from a redesigned synthetic gene by manipulating codons, because native genes have evolved for balanced expression of all cellular genes, not for maximal expression of any single gene. An optimized gene can improve protein expression from 2 to 10 fold, and in some cases more than 100 fold improvements have been reported (DNA 2.0, USA). Usually, codon optimization of a DNA segment can involve sequence modification without any changes in amino acid sequence (GeneScript, USA).

Software programs have been developed to perform automatic codon optimization (DNA2.0. Retrieved 11 May 2010). Optimum gene algorithms optimize a variety of parameters that are critical to the efficiency of gene expression, including: codon usage bias, Guanine and Cytosine nucleotides content, mRNA secondary structure, cryptic splicing sites, premature PolyA sites, internal chi sites and ribosomal binding sites, negative CpG islands, RNA instability motifs (ARE), repeat sequences (direct repeat, reverse repeat, and Dyad repeat) and restriction sites that may interfere with cloning (GeneScript USA). Several statistical methods have been proposed and used to analyze codon usage bias (Comeron and Aguade, 1998). Methods such as the 'frequency of optimal codons' (Fop) and the 'codon adaptation index' (CAI) are used to predict gene expression levels, while methods such as the 'effective number of codons' (Nc) and Shannon entropy from information theory are used to measure codon usage evenness (Peden, 2005). Multivariate statistical methods, such as correspondence analysis and principal component analysis, are widely used to analyze variations in codon usage among genes (Suzuki *et al*., 2008).

2.2 The Importance of Synthetic Genes in Plant Biotechnology

The application of synthetic genes instead of natural genes in plant biotechnology is developing rapidly. While the ability to make increasingly long stretches of DNA efficiently and at lower prices is a technological driver of this field. Attention is being focused on improving the design of genes for specific purposes. Nowadays, it is possible to assess of protein expression by synthetic genes for a purpose target and selection of appropriate organism or microorganism with highly expression of protein. For instance, in the case of the *bxn* gene the CAI (Codon Adaptation Index) before optimization is 0.65 and after optimization is 0.69 in *E.coli* (Figure 2; GeneScript, USA). The same sequence shows a CAI before optimization of 0.61 in tobacco, while after optimization this raises to 0.71 (Figure 3; GeneScript, USA). Therefore, the express of target protein is different in different organisms and this technique is a unique application of synthetic and optimized genes. Several reports are available based on using synthetic herbicide resistant genes such as synthetic *tfdA* gene resistant to herbicide 2, 4-D (Oliver *et al*., 2003). Using synthetic *cryIC* gene encoding a *Bacillus thuringiensis* δ-endotoxin protein confers *spodoptera* resistance has been reported in alfalfa and tobacco (Strizhov *et al.,* 1996). The transgenic expression of synthetic Bt genes are reportedly very effective for controlling insect pests in several major crop plants, including corn, rice, cotton, potato, tomato, tobacco, soybean and canola (Miklos *et al*. 2007). Codon optimization of *cry1Ab* gene has been reported for higher expression in plant organelles (Jabeen *et al*., 2010). It is expected that codon optimization will lead to higher expression levels of the *bxn*-encoded protein in different host organisms and cause more tolerance or rapid degradation of bromoxynil in plants. Therefore, using synthetic gene was very popular in some studies.



0.69

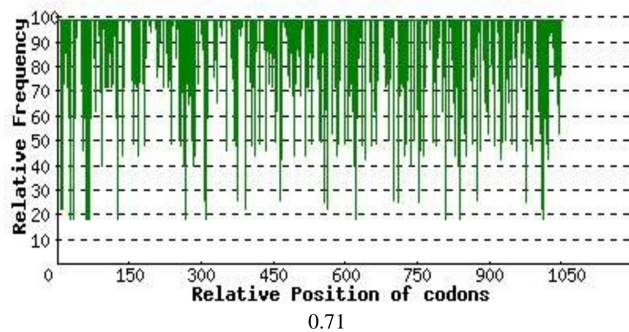
0.65

CAI (Codon adaptation Index)

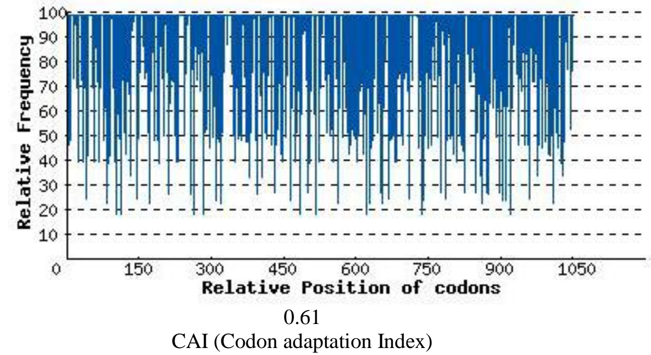
Figure 2 The distribution of codon usage frequency along the length of the gene sequence. The top is before optimization and the left is after optimum gene optimization for expression in *E.coli.* (A CAI of 1.0 is considered to be perfect in the desired expression organism, and a CAI of > 0.9 is regarded as very good, in terms of high gene expression level- Genescript, USA)

3.0 CONCLUSION

Researchers are able to manipulate codons to enhance protein expression level in synthetic genes. Therefore, with increasing of expressed protein level, the concern of limit protein expression via naturally occurred genes will be disappeared. In case of plants, it is possible to use synthetic herbicide or pesticide resistant genes as well as the genes that have been involved in resistant of crops to fungi, viruses and abiotic stresses. The synthetic and optimized *bxn* gene resistant to bromoxynil is a novel research project in genetic transformation of crops and it is proposed for future investigation. In this respect with regarding to the more advantages of utilization of synthetic genes can secure mass production of transgenic plants for future needs.



0.71



0.61

CAI (Codon adaptation Index)

Figure 3 The distribution of codon usage frequency along the length of the gene sequence. The top is before optimization (0.61) and the botom is after optimum gene optimization for expression in tobacco (0.71). (A CAI of 1.0 is considered to be perfect in the desired expression organism, and a CAI of > 0.9 is regarded as very good, in terms of high gene expression level- Genescript, USA)

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