Topolins: Current Research Status and Applications

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Article History

Manuscript No. 94
Received in 29th November, 2010
Received in revised form 24th January, 2011
Accepted in final form 26th February, 2011

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Keywords

Cytokinins, aromatic, biosynthesis, metabolism, structure-activity, micropropagation, yield, dormancy

Abstract

Topolins are hydroxylated analogues of 6-benzylaminopurine (BAP). They belong to a group of cytokinins with an aromatic side chain, in contrast to the isoprenoid group which contain an aliphatic side chain. Though topolins have been identified in diverse plant species, knowledge on their biosynthesis, signal transduction and metabolism is limited. Recent research advances in the isoprenoid group can therefore provide a template to direct further research on topolins. In spite of their high biological activity, applications of topolins to date have focused primarily on their use as an alternative source of cytokinin in micropropagation. The review starts with a primer on the history, nomenclature and chemical structure of topolins. A list of topolins identified in different plant species is provided to substantiate their widespread occurrence. A brief description of recent findings on the biosynthesis and metabolism of the isoprenoid group is offered to compare and contrast existing information on topolins. Determination of the biological activity of the stereoisomers of topolins is described as influenced by their chemical structure and signal perception. An account of the existing and plausible applications of topolins in yield accumulation, alleviating dormancy and abiotic stress management is provided. Finally, the review expresses confidence that topolins will play a significant role in the emerging scenario that cytokinins could hold the key to the next green revolution.

1. Introduction

The last decade has been very exciting for plant hormonal research with significant advances in our understanding of the existing hormones (Santner and Estelle, 2009), discovery of new plant hormones (Gomez-Roldan et al., 2008), exploring the network of hormonal cross-talk (Ross et al., 2001; Rashotte et al., 2005; Weiss and Ori, 2007; Chandler, 2009), and establishing the role of this network in key developmental processes (Ferguson and Beveridge, 2009; Subbaraj et al., 2010). This progress has been largely facilitated by genetic (McCourt, 1999; Ashikari and Matsuoka, 2002; Gazzarrini and McCourt, 2003), analytical (Birkemeyer et al., 2003; Pan and Wang, 2009) and bioinformatics (Peng et al., 2008) approaches.

Cytokinins are hormones with a characteristic trait of promoting cell division (Miller et al., 1955). In plants, they regulate diverse developmental and physiological processes such as the regulation of root and shoot growth, branching, chloroplast development, leaf senescence, stress response and pathogen resistance (Heyl and Schmülling, 2003). Two major groups of cytokinins are known. The naturally occurring adenine derivatives and the synthetic phenylurea group comprising CPPU and thidiazuron. The naturally occurring cytokinins are further divided into two groups based on the chemical structure of the adenine N^6-side chain as the isoprenoid group (ISCKs) which carries an aliphatic side chain, and the aromatic group (ARCKs) which carries an aromatic side chain (Mok and Mok, 2003). The ISCK group is mainly represented by Z and iP type of cytokinins, whereas the ARCK group is mainly represented by BAP and its hydroxylated analogues called topolins (Strnad, 1997).

Fundamental knowledge on the chemistry, activity and function of cytokinins was derived from early research on the ISCK group (Staden and Davey, 1979; Letham and Palni, 1983). New wisdom on the molecular mechanisms of biosynthesis (Kakimoto, 2003), perception and signal transduction (Heyl and Schmülling, 2003), metabolism and transport (Kudo et al., 2010) of cytokinins has also been provided by the ISCK group. Research on the ARCK group, though not equivalent to the ISCK group, has progressed mainly due to the exclusive techniques developed for this group of cytokinins, which includes the development of specific antibodies (Strnad, 1996), improved extraction and purification methods (Hoyerová

Abbreviations	
ABA	Abscisic acid
AHK	Arabidopsis histidine kinase
AMP/ADP/ATP	Adenosine mono/di/triphosphate
ARCKs	Aromatic cytokinins
BA3G/BA7G/ BA9G	6-benzylaminopurine 3-glucoside/6-benzylaminopurine 7-glucoside/6-benzylaminopurine 9-glucoside
BAP/BAR/ BAR5'P	6-benzylaminopurine/ 6-benzylaminopurine riboside/ 6-benzylaminopurine riboside 5'-diphosphate
CBP	Cytokinin binding protein
CKX	Cytokinin oxidase/dehydrogenase
CPPU	N-phenyl-N'- [2-chloro-4-pyridyl] urea
CRE1	Cytokinin response
CYP375A	Cytochrome P450 monooxygenase
cZ	cis-Zeatin
DHZ	Dihydrozeatin
DMAPP	Dimethylallyl pyrophosphate
DNA	Deoxyribonucleic acid
GA	Gibberellic acid
Gn1a	Grain number
HPLC	High performance liquid chromatography
iP/iPR/ iPRDP	Isopentenyl adenine/ Isopentenyl adenine riboside/ Isopentenyl ribose 5'-diphosphate
IPT	Isopentenyl transferase
ISCKs	Isoprenoid cytokinins
K/KR	Kinetin/kinetin riboside
LAD	Leaf area duration
LOG	Lonely guy
MemT/MemTR	Methoxy meta-topolin/ methoxy meta-topolin riboside
MeoT/MeoTR	Methoxy ortho-topolin/ methoxy ortho-topolin riboside
mRNA/tRNA	Messenger ribonucleic acid/transfer ribonucleic acid
MS	Mass spectrometry
mT/mTR/ mT9G/mTOG/ mTR5'P	Meta-topolin/meta-topolin riboside/ meta-topolin 9-glucoside/meta-topolin O-glucoside/ meta-topolin ribose 5'-diphosphate
oT/oTR/oT9G/ oTOG/oTR5'P	Ortho-topolin/ortho-topolin riboside/ ortho-topolin 9-glucoside/ortho-topolin O-glucoside/ortho-topolin ribose 5'-diphosphate
pT/pTR	Para-topolin/ para-topolin riboside
QTL	Quantitative trait loci
SAM	Shoot apical meristem
tZ/tZRDP	Trans-zeatin/ trans-zeatin ribose 5'-diphosphate
Z/ZR	Zeatin/ zeatin riboside

et al., 2006) and state-of-the-art HPLC and MS techniques (Tarkowski et al., 2009). These techniques have cumulatively enabled the identification of previously unidentified compounds of the ARCK group (Barciszewski et al., 2007; Huang et al., 2007).

Comparative studies on the signal perception (Spíchal et al., 2004), endogenous concentration (Baroja-Fernandez et al., 2002), metabolism (Alvarez et al., 2008) and bioactivity (Amoo et al., 2010) between ISCKs and ARCKs suggest that the structural differences between the two groups could have far reaching physiological implications than previously thought. Though progress has been made, clear gaps exist in our understanding of the role of topolins. The aim of this review therefore, is to provide an update on the current research status and applications of topolins with a perspective on potential areas of application.

2. History and Nomenclature

The isolation and identification of kinetin from herring sperm DNA (Miller et al., 1955), the first compound with cytokinin activity to be chemically identified (Skoog and Armstrong, 1970), sparked a race for the identification of compounds with similar activity in plants and the chemical synthesis of identical analogues (Skoog, 1994). While BAP was synthesized in the lab the same year as the discovery of kinetin (Skinner and Shive, 1955; Skoog, 1994), zeatin was identified as a natural cytokinin in corn (*Zea mays*) endosperm only eight years later (Letham, 1963).

For almost twenty years since its chemical synthesis, BAP and its analogues were considered to be synthetic, until a hydroxylated derivative of BAP was identified naturally in mature leaves of poplar (*Populus* × *robusta*) (Horgan et al., 1973). Subsequent identification of the same compound in fruits of *Zantedeschia aethiopica* (das Neves and Pais, 1980a) and its structural isomer in poplar (Strnad et al., 1997) with putative cytokinin-like activity in bioassays (Holub et al., 1998) provided substantial evidence for the prevalent occurrence of these type of compounds naturally. To distinguish these compounds from the existing ISCK group of cytokinins, the trivial name of 'populins' (from poplar) was adopted (Horgan et al., 1975). However, since populin was already in practice for another chemical salicin benzoate, the Czech name for poplar=topol, and therefore 'topolins' was adopted for the hydroxylated

analogues of BAP (Strnad, 1997).

Several systems of abbreviations have been proposed to unify the nomenclature of cytokinins and its myriad derivatives (Letham and Palni, 1983; Crouch et al., 1993; Kamínek et al., 2000). The convention used by Strnad (1997) shall be followed throughout this review, due to its simple representation of the positional isomers of topolins and their derivatives and due to the widespread usage of this system of abbreviations in topolin related literature. In this system, topolins are represented by the alphabet 'T'.

3. Chemical Structure

ARCKs (Figure 1a-d) possess an aromatic N^6-side chain, whereas the ISCKs (Figure 1e-h) possess an aliphatic side chain (Shaw, 1994). Topolins (Figure 1b-d) are differentiated from BAP (Figure 1a) by the presence of a hydroxyl group in the aromatic side-chain. Positional isomers of topolins are further differentiated by the presence of the hydroxyl group at the 2-(*ortho*), 3-(*meta*) or 4-(*para*) positions, and are thereby referred to as *ortho*-topolin (oT) (Figure 1b), *meta*-topolin (mT) (Figure 1c) and *para*-topolin (pT) (Figure 1d), respectively. In a few plants (Tarkowska et al., 2003), the presence of a methoxy group (OCH_3) at the *ortho*-(MeoT) and *meta*-(MemT) positions have also been reported.

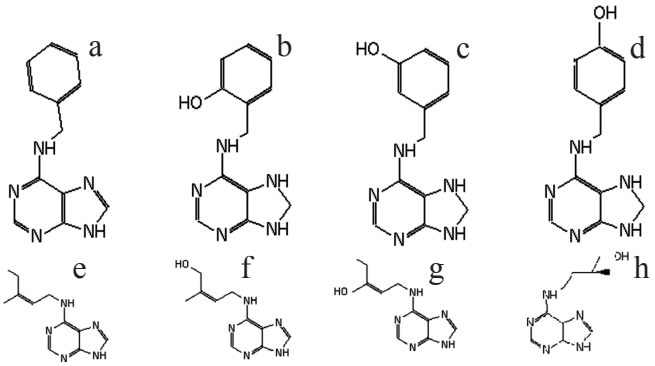


Figure 1: Chemical structures of key ARCKs and ISCKs represented by (a) BAP, (b) oT, (c) mT, (d) pT, (e) iP, (f) cZ, (g) tZ and (h) DHZ (From PMN: www.plantcyc.org)

Among the ISCKs, the zeatin group (Figure 1f-h) is distinguished from iP (Figure 1e) by the presence of a hydroxyl group in the side chain. The zeatin group is further categorized by the stereoisomeric position of the hydroxyl group as cZ (Figure 1f) where the hydroxyl group is present in the *cis* position, tZ (Figure 1g) where the hydroxyl group is present in the *trans* position, and DHZ (Figure 1h) where the hydroxyl group is saturated (Shaw, 1994).

4. Biosynthesis

At this point of time, knowledge on the biosynthesis of ARCKs is only speculative. In fact, credible knowledge on the biosynthetic pathway of ISCKs has been gathered only recently (Kakimoto, 2003). Two major modes of ISCK biosynthesis have been proposed: 1) via tRNA and 2) *de novo* synthesis of free cytokinins (Letham and Palni, 1983). In either mode, addition of the isopentenyl side-chain to an adenine base is considered to be the rate limiting step for cytokinin biosynthesis. The identification of the genes that encode the enzyme IPT, which catalyses this transfer in tRNA (Miyawaki et al., 2006; Sakamoto et al., 2006) and in the *de novo* pathway (Kakimoto, 2001; Sakamoto et al., 2006) has been crucial to the understanding of ISCK biosynthesis. tRNA-IPTs have been identified virtually in all organisms (Kamada-Nobusada and Sakakibara, 2009). However, the contribution of tRNA derived ISCKs to the total cytokinin pool is less than 40%, which suggests the significance of the *de novo* biosynthetic pathway (Kakimoto, 2003).

Detailed description of the *de novo* biosynthetic pathway of ISCKs has been dealt with elsewhere (Kakimoto, 2003; Kamada-Nobusada and Sakakibara, 2009). Briefly (Figure 2), considering ADP as a substrate for prenylation, IPT catalyses the transfer of the isopentenyl group from DMAPP to ADP

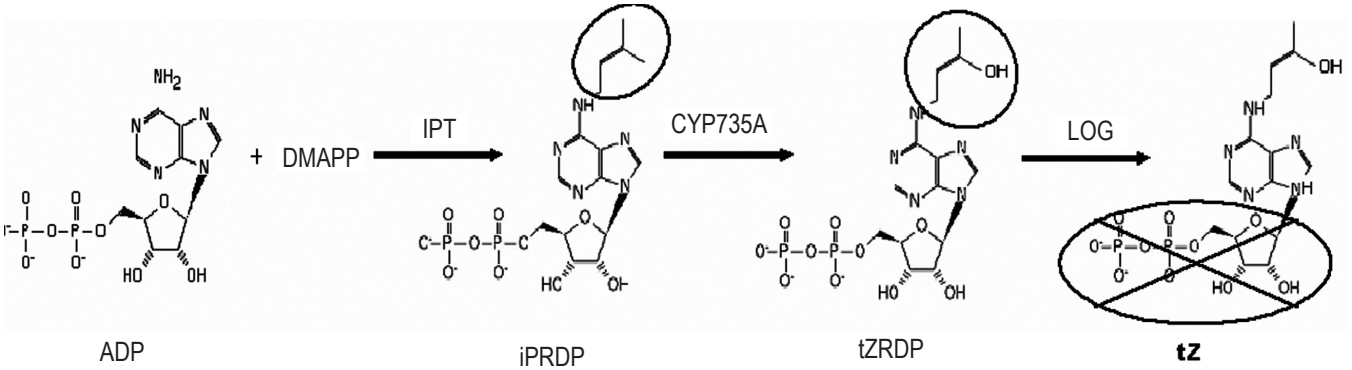


Figure 2: The proposed *de novo* biosynthetic pathway of ISCKs showing ADP as the preferred substrate for IPT to transfer the isopenetenly group from DMAPP resulting in the formation of iPRDP, followed by hydroxylation in the side chain catalysed by CYP735A resulting in tZRDP, and the release of the nucleotide group by the enzyme encoded by the LOG gene, finally resulting in the formation of tZ

resulting in the formation of iPRDP. The isopentenyl-nucleotide (iPRDP) then undergoes hydroxylation at the prenyl side-chain catalysed by CYP375A resulting in the formation of tZ-nucleotide (tZRDP). The *LOG* gene that encodes an enzyme with phosphoribohydrolase activity (Kurakawa et al., 2007) releases the ribose 5'-diphosphate moeity from tZ-nucleotide in the final step, resulting in the formation of tZ. ADP and ATP are the preferred substrates for prenylation in plants, whereas AMP is preferred by bacteria (Kakimoto, 2003).

Under the condition that no concrete evidence exists for the biosynthesis of ARCKs, it is not unreasonable to consider the *de novo* biosynthetic pathway of ISCKs as a template. With ADP/ATP as a substrate, a transferase enzyme may catalyse the transfer of a benzyl group to the N^6-position, resulting in BAR5'P. Subsequent hydroxylation of the benzyl side-chain, catalysed by CYP375A or its homolog may create the corresponding hydroxy benzyladenosine 5'-diphosphate, which ultimately results in the formation of topolins following the release of ribose 5'-diphosphate moeity by the enzyme encoded by the *LOG* genes or their homologues. Nevertheless, alternative biosynthetic pathways via tRNA or the metabolism of phenolics cannot be ruled out (Strnad, 1997).

5. Metabolism

Unlike the knowledge on biosynthesis, considerable information on the metabolism of ARCKs exists, largely derived from feeding experiments involving radiolabelled BAP (McCalla et al., 1962; Ramina 1979; Abo-Hamed et al., 1984; Zhang et al., 1987; Auer et al., 1992). However, substantial information on the metabolism of topolins per se is lacking, due to the limited studies that involve the tracking of radiolabelled topolins (Werbrouck et al., 1996). The metabolic events of cytokinins can be broadly classified into those involving 1) modifications of the adenine ring and 2) modifications of the side chain (Jameson, 1994).

5.1. Modifications of the adenine ring

5.1.1. Interconversion between the free base, nucleosides and nucleotides

The interconversion between the free base and their corresponding ribosides and ribotides/nucleotides (Figure 3) are features of adenine metabolism (Mok and Mok, 2003). Though the formation of the BAP ribotide (BAR5'P) is not a prerequisite for absorption in plants (Jameson, 1994), the biological activity of the free base is relatively higher than the corresponding ribosides and ribotides (Strnad, 1997). Given that the nucleotide acts as the substrate for cytokinin biosynthesis (Kakimoto, 2003), and the riboside and ribotide are capable of transport (Auer et al., 1992), this interconversion between the free base, nucleoside and nucleotides, could play a significant role in the

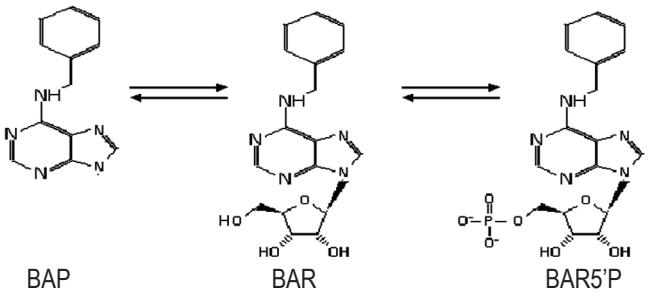


Figure 3: Forms of interconversion of cytokinins between the free base (BAP), the corresponding riboside (BAR) and ribotide (BAR5'P)

cytokinin turnover (Jameson, 1994).

5.1.2. Glucosylation

Glucosides are amongst the major metabolites of BAP (Werbrouck et al., 1995), and glucosylation can occur at the 3, 7 or 9 positions of the adenine moiety (Jameson, 1994) (Figure 4). Glucosides are considered to be storage forms of cytokinins (Strnad, 1997), and are therefore considered to be biologically inactive (Letham and Palni, 1983). The 9-glucoside is among the most prevalent metabolite of ARCKs identified in plants (Table 1).

5.1.3. N-alanyl conjugation

Conjugation of the amino acid alanine at the N^9-position of the adenine moiety has been detected as a metabolic product of both ISCKs (MacLeod et al., 1976) and ARCKs (Zhang et al., 1987). These metabolites not only possess enhanced stability, but are also capable of releasing free BAP, and are therefore considered biologically active (Jameson, 1994).

5.2. Modifications of the side chain

5.2.1. Hydroxylation

In the biosynthetic pathway of ISCKs (Figure 2), the addition and stereoisomeric position of the hydroxyl group determines the type of ISCK formed, which in turn determines its biological activity (Jameson, 1994). Likewise, it is logical to imagine a similar metabolic event for ARCKs, where hydroxylation at the *ortho*-, *meta*- or *para*- positions could determine the cor-

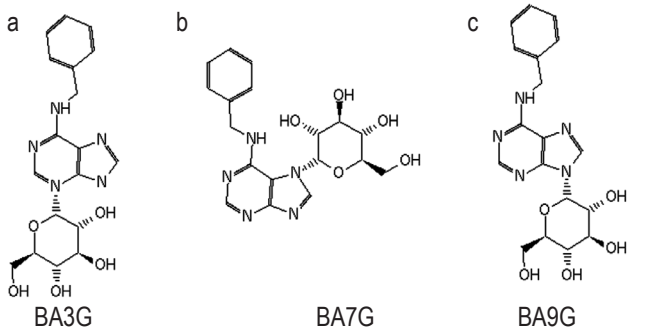


Figure 4: Derivatives of BAP showing glucosylation at the (a) N^3-, (b) N^7- and (c) N^9-positions of the adenine moiety

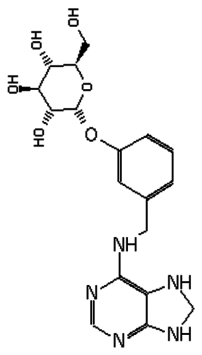
Table 1: Chronological order of the identification of ARCKs naturally in different plant species and plant parts			
Plant species	Plant part	ARCK identified	Reference
Poplar (*Populus* × *robusta* Schneid.)	Mature leaves	oTR	Horgan et al., 1973
Calla lily (*Zantedeschia* sp. K.*)*	Fruits	oTR, oT9G-2-methylthio analogue	Chaves das Neves and Pais, 1980a, b
Anise (*Pimpinella anisum* L.)	Cell culture	BAR	Ernst et al., 1983
Tomato (*Solanum lycopersicum* L.) plants infested with *Agrobacterium tumefaciens*	Crown galls of stems	BAP, BAR, BA9G	Nandi et al., 1989
Poplar	Young fully expanded leaves	oT, oTR, oT9G	Strnad et al., 1994
Oil palm (*Elaeis guineensis* Jacq.)	Shoots, inflorescence and embryos	BAP, BAR, BA9G, oT, oTR, oT9G, mT, mTR, mT9G	Jones et al., 1996
Poplar	Mature leaves	mT, mTR, mT9G	Strnad et al., 1997
Potato (*Solanum tuberosum* L.)	Leaf and stem	BAP, BAR, BA9G, oT, oTR, oT9G, mT, mTR, mT9G	Baroja-Fernandez et al., 2002
Chenopodium rubrum	Cell culture	oTOG, oTOG-2-methylthio analogue	Doležal et al., 2002
Arabidopsis (*Arabidopsis thaliana* L.) and poplar	Leaves	MeoT, MemT, MeoTR, MemTR	Tarkowská et al., 2003
Coconut (*Cocos nucifera* L.)	Immature inflorescence, SAM, spear leaf and embryo	BAP, BAR, BA9G, BAR5'P	Sáenz et al., 2003
Tagetes minuta	Dry achenes	BAP	Stirk et al., 2005
Gastrodia elata	Rhizomes	pTR	Huang et al., 2007
Beech (*Fagus sylvatica* L.)	Leaves and roots	BAP, BAR, BAR5'P, mT, mTR, mTR5'P, oT, oTR, oTR5'P	Winwood et al., 2007
Corn (*Zea mays* L.)	Xylem sap	BAP	Alvarez et al., 2008
Pea (*Pisum sativum* L.)	Roots	BAP, mT	Stirk et al., 2008

responding biological activity. However, in the feeding experiments conducted so far, the metabolism of radiolabelled BAP to any of the topolins has not been detected (Strnad, 1997). The detection of negligible amounts of free BAP in plants, suggests that the conversion of the BAP to its hydroxylated form could occur rapidly in plants (Strnad, 1997).

5.2.2. O-glucosylation

O-glucosides are abundant metabolites of *meta*-topolin and *ortho*-topolin capable of reversible sequestration (Strnad, 1997). In mT feeding experiments, mTOG (Figure 5) was a primary metabolite that degraded faster than BA9G, obtained with the application of BAP (Werbrouck et al., 1996). O-glucosides have been shown to hydrolyse during key developmental stages of plants, probably resulting in formation of the free bases (Jameson, 1994; Strnad, 1997).

Apart from the metabolites discussed above, other derivatives comprising modifications in the adenine and side chain moiety have been identified naturally in plants (Table 1). A 2-methylthio analogue of oT has been identified in fruits of *Zantedeschia aethiopica* (das Neves and Pais, 1980b) and methoxy derivatives of oT and mT have been identified in *Arabidopsis* and poplar (Tarkowská et al., 2003). Their significance in the metabolic events of topolins is yet to be determined. Considering the scope of this review, the main metabolic features of topolins have been briefly described here. A more elaborate description of the metabolic events of ARCKs has been described by Strnad (1997), and detailed analyses of the metabolism of ISCKs (Letham and Palni, 1983; Jameson, 1994; Mok and Mok, 2003) and the enzymes involved (Mok



mTOG

Figure 5: Derivative of mT showing O-glucosylation in the side chain moiety

and Martin, 1994) have been discussed elsewhere.

6. Natural Occurrence

Natural occurrence of topolins is not confined to plants. They have also been identified in lower order plants such as the moss *Physcomitrella patens* (Von Schwartzenberg et al., 2007) and sea-weed extracts (Stirk et al., 2003). Though feeding experiments using radiolabeled BAP (Strnad, 1997) and mT (Werbrouck et al., 1996) have provided several metabolites, wider knowledge on the derivatives of topolins has been obtained from the identification of topolins and/or their metabolites naturally in different plant species and plant parts (Table 1). The identification of topolins and its metabolites in a wide range of plant species (Table 1) suggests that the natural occurrence of ARCKs could be more widespread than previously imagined. Their identification in a range of plant parts also suggests that either these parts are capable of *de novo* synthesis and/or ARCKs or their precursors are capable of translocation.

Widespread occurrence of cytokinins was further corroborated by the expression of *Arabidopsis* IPT genes in several plant parts (Kudo et al., 2010). The conviction however, is that the roots are the primary site of biosynthesis (Staden and Davey, 1979; Letham, 1994). The spatial distribution of cytokinins may therefore occur due to a circulatory transport mechanism involving translocation via the xylem and phloem (Staden and Davey, 1979; Stirk and Van Staden, 2010). Accordingly, different isoforms of cytokinins are present in the xylem and phloem. Zeatin type ISCKs are predominantly found in the xylem, whereas the iP type is predominantly found in the phloem (Romanov, 2009). While the cross-talk between the different isoforms is ambiguous at this stage, the predominant expression of CYP375A in the roots (Takei et al., 2004), localization of *LOG* mRNA in the shoot meristem tips (Kurakawa et al., 2007), and the differential perception of the cytokinins by the receptors (Spíchal et al., 2004) suggest that elucidation of the different delivery mechanisms of cytokinins is still required to fully understand their role in plant development.

In general, the free bases, ribosides and ribotides are considered to be motile in comparison to the glucosides (Auer et al., 1992; Strnad, 1997). Due to the lack of sufficient feeding experiments and knowledge on the biosynthetic pathway of topolins, the mechanisms of transport remain unknown. However, radiolabelled tracking experiments have shown that BAP is capable of acropetal (Mozes and Altman, 1977) and basipetal (Black and Osborne, 1965) transport. This possibly explains the natural occurrence of a wide array of topolins in different plant species and different plant parts (Table 1).

7. Structure-Activity Relationship

A compound is suggested to possess cytokinin function largely based on the results of bioassays (Mok, 1994). Therefore, the identification of a cytokinin either naturally (Horgan et al., 1975; Strnad et al., 1994, 1997) or through chemical synthesis in the lab (Skoog et al., 1967; Doležal et al., 2007) is immediately followed by the description of its activity in bioassays. The most common bioassays used to determine cytokinin activity are the tobacco callus bioassay, wheat leaf senescence bioassay and the *Amaranthus* bioassay (Kaminek et al., 1987a; Holub et al., 1998; Doležal et al., 2007), though other bioassays such as the radish leaf expansion bioassay (Letham, 1971), soybean callus bioassay (Miller, 1961), and the release of lateral buds of peas from apical dominance (Kaminek et al., 1987a) have also been described. While the bioassays provide preliminary evidence of the biological activity of cytokinins, the sensitivity of bioassays could vary for different cytokinins (Holub et al., 1998; Sakakibara, 2006). Careful interpretation of the results from bioassays is therefore advised. The biological activity of cytokinins is determined by 1) chemical structure of the cytokinin comprising modifications in the side chain and the adenine ring, and 2) perception of the cytokinin signal by the receptors and subsequent initiation of the down stream reactions.

7.1. Chemical structure

7.1.1. Modifications of the side chain

Cytokinin activity is determined by the presence or absence of the hydroxyl groups and their stereoisomeric position in the side chain moiety (Sakakibara, 2006). Between iP and Z, Z with a hydroxyl group in the side chain was found to be at least 5 times more active than iP which lacks the hydroxyl group in a soybean callus bioassay (Manos and Goldthwaite, 1976). Within the Z group, the presence of the hydroxyl group in the *trans*-position in tZ showed higher biological activity in many bioassays than cZ, in which the hydroxyl group is present in the *cis*-position (Mok and Mok, 2003). Likewise in ARCKs, the presence of the hydroxyl group in topolins could render higher biological activity than BAP, and within the topolins, the stereoisomeric position of the hydroxyl group in the *ortho*/*meta*/*para* positions could determine the biological activity.

The high biological activity of hydroxylation at the *meta*-position of the aromatic side chain was revealed (Horgan et al., 1975) even before the identification of mT naturally (Strnad et al., 1997). In the tobacco callus and wheat leaf senescence bioassays, mT showed higher cytokinin activity than BAP and oT, and was on par with that of Z. However, in the more sensitive *Amaranthus* bioassay, the activity of mT was less than that of Z and BAP at their highest concentrations. Nevertheless, hydroxylation at the *para*- and *ortho*-positions consistently showed low biological activity in all the bioassays (Kaminek et al., 1987a; Holub et al., 1998).

The presence of the hydroxyl group in the side chain facili-

tates the formation of O-glucosides (Werbrouck et al., 1996), which are capable of rapid conversion to the free base when required (Strnad, 1997). oTOG, identified naturally in suspension cultures of *Chenopodium rubrum* (Doležal et al., 2002), showed only moderate biological activity in the *Amaranthus* bioassay, probably due to the conjugation in the *ortho*-position of the side chain. The methoxy derivatives of topolins (MeoT and MemT) identified naturally in *Arabidopsis* and poplar showed biological activity on par with BAP in the tobacco callus and *Amaranthus* bioassays. However, in the wheat leaf senescence bioassay, their activity was twice higher than that of BAP (Tarkowská et al., 2003).

7.1.2. Modifications of the adenine ring

An intact adenine ring is required for high cytokinin activity (Skoog et al., 1967), and therefore the free bases possess higher cytokinin activity than their corresponding metabolites (Sakakibara, 2006). Amongst the modifications of the adenine ring, ribosides and the 9-glucosides of topolins have been frequently identified naturally in different plant species (Table 1). Ribosylation of Z at the 9-position caused a 10-fold reduction in biological activity (Holub et al., 1998). A comparison of oTR, mTR and pTR with BAR, showed that oTR and pTR always exhibited low biological activity, whereas mTR showed high activity in all the bioassays tested (Kaminek et al., 1987a). Also, the free base mT showed higher biological activity than mTR in the *Amaranthus* bioassay, whereas their activity was the same in the wheat leaf senescence test (Holub et al., 1998). mTR and BAR showed higher biological activity than ZR in the tobacco callus bioassay (Holub et al., 1998), whereas MemTR showed higher activity than mTR and mT in the soybean callus bioassay (Amoo et al., 2010).

The general trend of the biological activity in the three bioassays was *meta*≥*ortho*≥*para* (Doležal et al., 2007). However, irrespective of the side chain, glucosylation at the 9-position of the adenine ring exhibited biological activity near zero in ARCKs (Holub et al., 1998) and poorer than the control in ISCKs (Letham and Palni, 1983).

7.2. Perception and signal transduction

The first step in the chain reaction that ultimately manifests as a biological response is the perception of the signal. The difference in the relative activities of different cytokinins in bioassays could therefore be due to the perception and downstream signal transduction processes. Three cytokinin receptors, CRE1/AHK4, AHK3 and AHK2, were identified in *Arabidopsis* (Inoue et al., 2001; Suzuki et al., 2001; Yamada et al., 2001) and the signal transduction of cytokinin was found to occur through a two-component signaling system involving a phosphorelay, prominent in bacteria and similar to the signal transduction of ethylene (Hwang and Sheen, 2001). A detailed discussion of cytokinin signaling can be found elsewhere (Heyl and Schmülling, 2003; Werner and Schmülling, 2009; Keiber and Schaller, 2010). Most bioassays measure cytokinin activity over a long duration, while cytokinins are capable of rapid degradation. A bacterial system to determine cytokinin activity rapidly was therefore developed, which involves expression of the cytokinin receptors in *E. coli* (Suzuki et al., 2001; Yamada et al., 2001).

In this system, ISCKs had higher activity than ARCKs in both CRE1/AHK4 and AHK3 receptors expressed in *E. coli*. Among the ARCKs, mT showed highest activity in both the receptors, with about 30% activity in CRE1/AHK4 and 80% activity in AHK3 (tZ showed 100% activity in both receptors), followed by BAP which showed 7.7% activity and 23.6% activity in CRE1/AHK4 and AHK3 respectively, and oT which showed no activity whatsoever. The corresponding ribosides showed no activity in CRE1/AHK4, whereas in AHK3 the activity was in the same order as the free bases. The O- and N-glucosides of ISCKs tested in this system also showed no activity with both the receptors (Spíchal et al., 2004).

Testing these compounds *in planta* using a reporter gene-based bioassay in *Arabidopsis* (D'Agostino et al., 2000) however corroborated the results obtained from bioassays. mT and BAP showed high activity on par with tZ, with oT and the corresponding ribosides expressing relatively less activity. O-glucosides showed high activity in this assay, probably because of breakdown to the free base *in planta*. This assay however integrates responses from several cytokinin pathways, and therefore cannot provide conclusive evidence on cytokinin activity (Spíchal et al., 2004). Based on the variety of results obtained with ARCKs in different bioassays, between the two receptors tested in the bacterial system, and the reporter gene-based bioassay, it seems that ARCK activity involves unidentified mechanisms. This could possibly involve exclusive sensing mechanisms for ARCKs (Doležal et al., 2007) which could belong to a group of unidentified CBPs (Keim and Fox, 1980; Strnad, 1997), or the involvement of the receptor AHK2 which was not tested in the bacterial system (Spíchal et al., 2004).

8. Applications of Topolins

8.1. Cytokinin source in micropropagation

The global market potential for tissue culture plants is expected to be worth 15 b US$ annum^{-1} (Govil and Gupta, 1997), and the major share of this volume is contributed by plants for cut flowers and pot plants (Prakash, 2007). Since its chemical synthesis, BAP has been the most preferred cytokinin component of the tissue culture industry to stimulate shooting in vitro, owing to its high activity and affordable price (Werbrouck et al., 1995). However, BAP has several inherent drawbacks which includes inhibition of root formation (Werbrouck et al.,

1995), high abnormality index (Amoo et al., 2010) and non-maintenance of histogenic stability (Bogaert et al., 2004). This leads to acclimatization problems and poor regeneration ex vitro, resulting in high production costs (Prakash, 2007). These problems of the tissue culture industry can partly be resolved by using alternative sources of cytokinins. Topolins were therefore tested as viable alternatives for BAP in the micropropagation of *Spathiphyllum* sp. (Werbrouck et al., 1996). Since then they have been used successfully for the micropropagation of several plant species such as turmeric (Salvi et al., 2002), potato (Baroja-Fernandez et al., 2002), plantain (Roels et al., 2005), *Aloe* (Bairu et al., 2007), banana (Bairu et al., 2008) and sea oats (Valero-Aracama et al., 2010).

The inhibition of root formation by BAP during the micropropagation of *Spathiphyllum* sp. was attributed to the formation of BA9G in the basal part of the callus, which remained unmetabolized for several weeks (Werbrouck et al., 1995). On the contrary, when mT was used, root formation was not inhibited and the O-glucoside of mTR was the major metabolic product (Werbrouck et al., 1996). Localized accumulation of mTR is avoided by rapid translocation to other plant parts, and the presence of the riboside group could prevent glucosylation at the 9-position (Kaminek et al., 1987a). The O-glucoside is also capable of sequestration to its free base when required in contrast to the 9-glucoside which is a storage form (Strnad, 1997). During the micropropagation of *Barleria greenii* (Amoo et al., 2010), BAP reported the highest abnormality index measured as a ratio of abnormal adventitious shoots to normal ones, whereas all the topolins tested (mT, mTR and MemTR) had an abnormality index lower than that of the control. BAP has been reported to cause phytotoxicity in plants (Bogaert et al., 2004), also probably due to the sessile nature of BA9G (Amoo et al., 2010). Maintenance of the histogenic composition of leaves is essential during the micropropagation of chimeras. In a *Petunia* leaf-variegated chimera (Bogaert et al., 2004), MemTR was superior to BAP in the maintenance of histogenic composition, and produced only a small number of albinos and green shoots than BAP. Other benefits of topolins in micropropagation include their anti-senescence activity in *Rosa* shoots in vitro (Bogaert et al., 2004) and alleviation of hyperhydricity of *Aloe* shoots in vitro (Bairu et al., 2007).

Topolins have overcome some of the serious drawbacks inherent to BAP, the traditional cytokinin source in micropropagation for more than fifty years. This advantage of topolins combined with increased shoot production in vitro (Amoo et al., 2010) provides an effective alternative source of cytokinins for the micropropagation industry. However, the beneficial effects of topolins are not universal. In *Vaccinium* sp. (Meiners et al., 2007) and azaleas (Mertens et al., 1996), tZ was superior to mT, and in wild service tree (Malá et al., 2009), BAP was better than mT and MemTR in promoting adventitious shoot production. Also, mTR was shown to inhibit rooting in some plant species (Werbrouck, 2008). Further research on the metabolic properties of topolins and extensive evaluation in other plant species is suggested. Albeit, the choice of cytokinins could determine the success or failure of the micropropagation of a plant species (Werbrouck, 2008), and topolins are very promising in this area of application.

8.2. Increase in agricultural yield/biomass

Manipulation of the genes that regulate GA biosynthesis (Salamini, 2003) resulted in the development and widespread adoption of short and sturdy dwarf varieties of wheat and rice, characterized by resistance to lodging by wind and rain and effective in utilization of fertilizers to produce high yield, which led to the green revolution (Borlaug, 1983). The global population has multiplied several folds since then, and is expected to reach 8.9 b by 2050. To feed this ever increasing population, the productivity of food crops has to increase by 50% (Sakamoto, 2006).

Cytokinins influence several yield related components such as the delay of senescence, resistance of plants to various forms of stress, respiration, source-sink activity and alleviation of apical dominance (Kamínek, 1992). Exogenous application of cytokinin therefore increased the yields of corn, rice, pepper, cucumber and cantaloupe (Mayeux et al., 1983). In cereals, exogenously applied topolins increased the grain weight plant^{-1}, elongation of the ears, number of grains head^{-1}, weight of grain head^{-1}, leaf area, leaf area duration and accumulation of nutrients in the grains, thereby increasing the harvest index of the plant (Hradecká and Petr, 1992; Trčková et al., 1992).

The net photosynthesis of a plant bears a direct relationship to the biomass produced, which in turn alters the source-sink allocation within the plant, ultimately influencing the harvest index of the plant (Sakamoto, 2006). Cytokinins are well known to regulate photosynthetic processes of the plant, either directly by increasing chlorophyll synthesis (Kuraishi et al., 1992) and/or indirectly by mediating light responses (Argueso et al., 2009). Winter rye plants treated with mTR had increased leaf area and LAD (Hradecká and Petr, 1992). In geophytes such as sugar beet, foliar application of mTR delayed the senescence of leaves by increasing the chlorophyll content and the net photosynthetic rate of the leaves. This, possibly by altering the source-sink distribution, resulted in increased root biomass and hence total biomass of the plant (Čatský et al., 1996).

The formation of O-glucoside in preference to N-glucosides (Werbrouck et al., 1996), combined with rapid translocation (Kaminek et al., 1987a) and degradation of the corresponding metabolites (Bairu et al., 2008) could provide topolins the advantage of being used to increase agricultural yield in prefer-

ence to BAP. Recently, a QTL that increases grain number, *Gn1* was identified in rice (Ashikari et al., 2005). It was found to encode a gene for CKX, which irreversibly degrades cytokinins (Mok and Mok, 2003). Transgenic rice plants with antisense CKX genes had low levels of CKX, resulting in an increase in grain number (Ashikari et al., 2005). CKX is selective in identifying substrates for degradation. ISCKs with a double bond in the side chain are preferred substrates for catalytic cleavage of the side chain by CKX, compared to ARCKs and O-glucosides (Strnad, 1997). This immunity of ARCKs to CKX degradation could have a major impact on the cytokinin pool of the plant, and ARCKs could be directly involved in increasing the grain number of transgenic *Gn1* plants.

Cereals and geophytes constitute the major volume of food crops. Given that the exogenous application of topolins influences the yield parameters of cereals and geophytes, the results described above are indicative of the potential application of topolins. However, very few studies have reported the influence of topolins on yield related parameters. Further research in this area is recommended.

8.3. Alleviating dormancy

Dormancy is the temporary suspension of visible outgrowth of any structure containing a meristem and can be caused by factors outside the bud (para-dormancy/apical dominance), intrinsic factors within the bud (endo-dormancy) or environmental factors (eco-dormancy) (Lang, 1987). The inception and release of dormancy, which comprises overlapping phases of para- and/or endo- and/or eco-dormancy, involves decision making in the SAM orchestrated by hormonal cross-talk (Subbaraj et al., 2010). Cytokinins are historically known to stimulate branching by alleviating apical dominance (Sachs and Thimann, 1967). This process is primarily controlled by an antagonistic relationship with auxin, mainly synthesized in the apical bud, capable of regulating the local synthesis of cytokinins (Tanaka et al., 2006) and/or cytokinin export from the roots (Bangerth, 1994). Cross-talk with other hormones such as GA (Weiss and Ori, 2007) and strigolactones (Ferguson and Beveridge, 2009) also plays a vital role in the control of branching.

This ability of cytokinins to stimulate branching is of great use to increase the production of propagules or cuttings for many plant species in commercial nurseries. Exogenous application of mTR was more effective than BAP in increasing the number of cuttings in *Poinsettia* and *Gerbera*. The detrimental effects of BAP such as the inhibition of root formation and phytotoxicity were not observed following the application of mTR (Kaminek et al., 1987b). Floral productivity of calla lilies is directly related to the number of branches plant^{-1}. Therefore, the stimulation of branching by the exogenous application of BAP could have commercial implications (Subbaraj et al., 2010).

The role of cytokinins in the control of endo-dormancy is still poorly understood (Horvath, 2009). After the onset of endo-dormancy, BAP failed to stimulate branching in calla lilies (Subbaraj et al., 2010), whereas in potato, exogenous application of cytokinin released buds from endo-dormancy (Turnbull and Hanke, 1985). The establishment of endo-dormancy could coincide with a decline in the sensitivity of the buds to cytokinins (Subbaraj et al., 2010) and/or a change in the endogenous cytokinin concentration (Turnbull and Hanke, 1985). The endogenous cytokinin pool of potato cv. 'Kennebec', a cultivar which expressed high viability in vitro largely comprised ARCKs (92%), in contrast to cv. 'Jaerla', a cultivar with low viability, which comprised up to 57% of ISCKs (Baroja-Fernandez et al., 2002). Alleviating bud dormancy of food crops, by stimulating branching/tillering and extending the period of active growth, can have an indirect influence on the yield/biomass, and topolins have a definite role in the control of dormancy.

8.4. Management of abiotic stress response

Cytokinins are mainly synthesized in the roots (Letham, 1994) and are involved in signaling water status and available nutrient information or the lack of it to the shoot (Argueso et al., 2009). Therefore, under conditions of abiotic stress, for example drought, cytokinin signaling aids in retaining the photosynthetic capacity of the shoot (Werner and Schmülling, 2009). One of the initial reactions on the exposure of plants to drought conditions is enhanced CKX activity (Havlova et al., 2008) causing a reduction in ISCK content in the xylem sap (Schachtman and Goodger, 2008). ABA, which controls stomatal conductance of leaves (Davies et al., 2005) is also synthesized in the roots, and was found to increase CKX gene expression (Brugiere et al., 2003). Concomitant with the decline in endogenous ISCK level, a surge in endogenous BAP content was noticed in drought induced maize plants (Alvarez et al., 2008). As mentioned earlier, the CKX activity by reducing the concentration of ISCKs, could allow the ARCKs to be directly involved in retaining the photosynthetic activity of drought induced plants.

The cytokinin signaling pathway is also affected by cold and salt stress conditions. Cold stress rapidly down regulated the expression of the three cytokinin receptors. While *AHK2* and *AHK4* were down-regulated in *Arabidopsis* plants exposed to osmotic or salt stress, *AHK3* was up-regulated (Argueso et al., 2009). Such changes in cytokinin receptor activity have also been described in other plants. Therefore, apart from the regulation of the endogenous cytokinin pool, receptor activity could also be regulated in response to abiotic stress. How topolins manage abiotic stress control under the emerging scenario of climate change and efficient water use, is a topic worth further

investigation.

8.5. Other applications

Floral transition of *Sinapis alba* was marked by a transient increase in the influx of endogenous cytokinins to the shoot, which may probably be used for the subsequent formation and differentiation of floral organs (Lejeune et al., 1994). However in *Chenopodium rubrum*, floral differentiation was inhibited by the application of BAP (Blažková et al., 2001). Visible manifestation of flowering could involve factors beyond the control of cell division. While BAP did not stimulate the differentiation of floral primordia in calla lilies, it facilitated the visible manifestation of floral initiation stimulated by GA (Subbaraj et al., 2010). By promoting cell division and the ensuing DNA synthesis, the application of topolins directed the initiation of germination in seeds of *Tagetes minuta* (Stirk et al., 2005), and also enhanced the subsequent cotyledon growth (Palavan-Ünsal et al., 2002). Physiological and morphological changes in response to biotic stress are mediated by cytokinins, which involves changes in the cell division pattern caused by the pathogen/symbiont. Several microbes such as *Erwinia herbicola*, *Pseudomonas syringae*, *Rhodococcus fascians*, *Plasmodiophora brassicae* use this mechanism to infect host plants (Werner and Schmülling, 2009). In the classic plant-pathogen interaction involving *Agrobacterium tumefaciens*, BAP and its derivatives were identified in the crown galls of infested tomato plants (Nandi et al., 1989). The role of cytokinins in the nodulation process of nitrogen-fixing bacteria is also being investigated (Argueso et al., 2009).

That topolins have functional roles beyond cell division control was hypothesized even at the time of its discovery, when Horgan et al. (1975) detected oTR in mature, fully expanded leaves of poplar that had ceased cell division. The transient increase in topolin levels on exposure to light was suggestive of a role in leaf expansion (Hewett and Wareing, 1973).

In plant tissue culture, cytokinins have the ability to induce callus growth, a group of undifferentiated cells that proliferate incessantly in a disorganized manner. Since this mechanism is similar to that of cancer in animal cells, a role for cytokinin in the control of cancer was suggested (Doležal et al., 2007). This was followed by reports that demonstrated the ability of cytokinin bases to induce cell differentiation in human cancer cells (Ishii et al., 2003). Consequently, numerous derivatives of both ISCKs and ARCKs were chemically synthesized to test their ability as anti-cancer drugs (Doležal et al., 2006, 2007). Ribosides were more effective in regulating cancer formation at lower concentrations than the free bases (Voller et al., 2010). While the anti-cancer activity of iPR, KR and BAR was confirmed, oTR which showed minimal biological activity in plant bioassays (Kaminek et al., 1987a; Holub et al., 1998), had the highest anti-cancer activity in a panel of cancer lines tested (Voller et al., 2010).

9. Conclusion

The early identification of the ISCK group of cytokinins naturally (Letham, 1963), prior to the ARCK group (Horgan et al., 1973), has been advantageous for the progress of research in the ISCK group. Nevertheless, recent advances in the elucidation of the biosynthesis (Kakimoto, 2003), metabolism and transport (Kudo et al., 2010), and signal transduction (Heyl and Schmülling, 2003) of the ISCK group has provided an inventory of information to compare and contrast the existing information on ARCKs. Therefore, an account of the current research status of the ARCK group was assumed to be timely and beneficial to identify further areas of research. In spite of its high biological activity (Holub et al., 1998), the application of topolins in plant science has primarily focused on its use as an alternative cytokinin source in micropropagation, with a few reports on enhancing yield parameters (Hradecká and Petr, 1992; Trčková et al., 1992), and stimulation of branching (Kaminek et al., 1987b). This review was therefore aimed to provide a compilation of existing information on topolins with simultaneous reference to the recent developments in ISCK research, and to propose potential areas of application.

The identification of topolins in a broad range of plant species suggests that the natural occurrence of this type of cytokinins is more widespread than initially thought. Though the biosynthetic pathway of ISCKs seems to be an ideal template for ARCK biosynthesis, where the key enzymes in ISCK synthesis could play similar roles in ARCK synthesis, the lack of identification of the basic components such as the substrate and side chain donor, suggest an exclusive biosynthetic pathway for ARCKs. The relative susceptibility of ISCKs to CKX degradation (Strnad, 1997) and the metabolism of topolins to O-glucosides in preference to the N-glucosides of BAP (Werbrouck et al., 1995, 1996) highlight the significance of structural differences between the ISCK and ARCK groups and within the ARCK group, respectively. The differential perception of the ISCK and ARCK groups by the cytokinin receptors also corroborates an exclusive sensing and signal transduction strategy for ARCKs (Spíchal et al., 2004). Overall, key aspects of the biosynthesis, metabolism and signal transduction of ARCKs still remain ambiguous, and require further investigation.

As mentioned earlier, studies on the applications of topolins are limited, and have been confined to its recommendation as an alternative source of cytokinin to BAP in micropropagation (Amoo et al., 2010). In other plant developmental processes, topolins are either reported to play a direct (Kaminek et al., 1987b; Trčková et al., 1992) or indirect role (Ashikari et al., 2005; Alvarez et al., 2008) by affecting the endogenous cytokinin pool. Overall, this review expresses confidence that topolins

have wider physiological roles than currently reported.

10. Perspective

A better understanding of the vital aspects of ISCK biosynthesis and signal transduction has led to the development of genetically engineered transgenic plants involving key biosynthetic/metabolic genes. A list of the transgenic plants developed and the cytokinin genes involved have been listed by Ma (2008). Mostly, this involves enhanced cytokinin production via overexpression of IPT genes (Kakimoto, 2003) or silencing the CKX gene (Ashikari et al., 2005). The resulting transgenic plants have been assessed for their effects on cytokinin mediated developmental processes such as seed production, leaf and flower anti-senescence, stress adaptation and fruit development (Ma, 2008). While the use of such transgenic plants has several benefits, over production of cytokinins could have detrimental effects on plants such as phytotoxicity. Either spatial and/or temporal expression of the respective genes or application of cytokinins as a chemical spray at the desired concentration and stage of growth of the plant would enable controlled cytokinin action.

The diminishing land area available for agriculture combined with the burgeoning global population has underlined the need to improve the productivity of food crops. Cytokinins are known to affect several yield related parameters of plants. While regulation of GA synthesis was employed for the first green revolution, scope for the next green revolution relies largely on the use of cytokinins (Sakakibara, 2006). In that emerging scenario, the salient features of topolins such as their high biological activity, easy chemical synthesis at affordable prices and minimal deleterious effects on plants, would establish them as the ideal source of cytokinins.

11. Acknowledgements

I dedicate this review to Professor Miroslav Strnad and his team for their pioneering research on topolins.

I thank the Ministry of Education, Government of New Zealand for funding my PhD and Dr. Panimalar Vijayan for constructive suggestions.

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