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Tracking the Story of Cytokinin Research

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Abstract Existence of compound(s) inducing cell division in excised plant tissues was spotted more than 100 years ago. Since then research of cytokinins (CKs), plant hormones which in cooperation with other phytohormones, namely auxin, control cytokinesis, and a number of other physiological processes in plants, has advanced correspondingly to the progress in other fields of life sciences. This historical overview is focused on major topics of CK research including (1) discovery of CKs, (2) search for natural CKs, (3) role of CKs in transfer RNA, (4) biosynthesis (5) metabolism, (6) signaling of CKs, and (7) molecular probing of the physiological functions of CKs. Some parts of these subjects can already be assessed within the context of an appropriate time span necessary for critical evaluation. I have used this opportunity to present also some personal recollections, namely those of Prof Folke Skoog, in whose laboratory the first CK, kinetin, was discovered.

Keywords Cytokinin · History · Plant hormones · t-RNA · Cytokinin biosynthesis · Cytokinin metabolism

Introduction

Oats, peas, beans and barley grow,
Oats, peas, beans and barley grow,
Can you, or I, or anyone know
How oats, peas, beans and barley grow?
(Old English Nursery Rhyme quoted by Went and Thimann in "Phytohormones", 1937)

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Monitoring the history of research in a special field of plant science represents a great adventure. It allows confrontation of the presumptions and speculations of scientists about the role of what at that time were new findings and the attempts of these scientists to fit their discoveries into proposed functional system(s). In contrast to reviews focused on the actual state of knowledge, the historical review gives us an opportunity to appreciate the power of intellectual factors in the process of uncovering the secrets of plant life.

Cytokinins (CKs) are a distinguished class of plant hormones that in interaction/cooperation with other hormones, especially auxin, control cell division, and a number of other fundamental biological processes, including lateral shoot formation, leaf senescence, formation and strength of metabolic sinks, and others (see Mok 1994). Those occurring naturally are adenine derivatives bearing either an isoprenoid or a ring substituent at the N^6 position (Fig. 1). Uncovering various aspects of CK formation, action and functions in plants has been a long-distance run, requiring application of various strategic and methodological approaches. Taking this into account, as well as being limited by available space, this essay is focused only on major topics of CK research. Some parts of these subjects can already be assessed within the context of an appropriate time span necessary for critical evaluation. I have used this opportunity to present also some personal recollections, namely those of Prof Folke Skoog. These are not always related to science, but they may contribute to an understanding of this interesting person.

The story of CK research involves a long period of presentiment of their existence, followed by identification of the chemical structures of kinetin and the first natural CK, *trans*-zeatin (*trans*Z). Biological functions of CKs have been gradually recognized and since the chemical

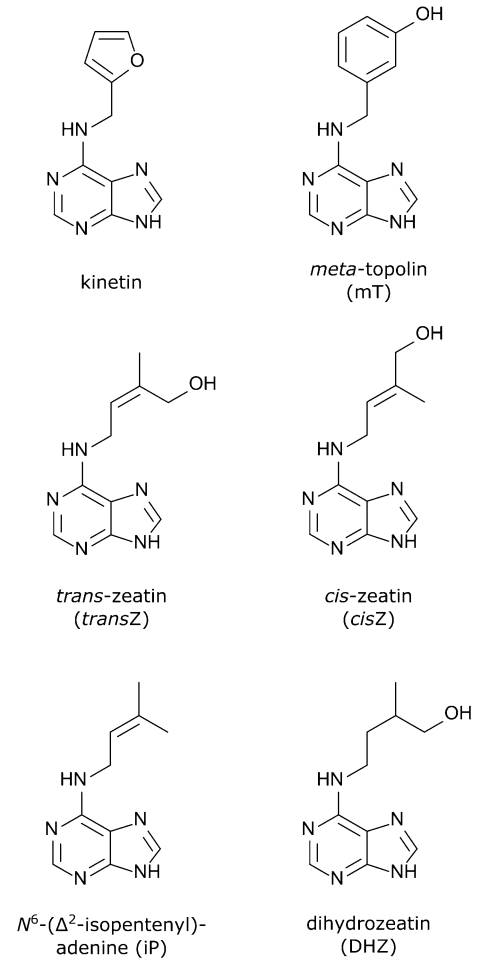

Fig. 1 Structures of kinetin and basic natural cytokinins

nature of CKs was determined, their biosynthesis, metabolism, translocation, and signaling in plants have been to a great extent resolved. Moreover, the research of CKs provided a number of direct or associated applications, not only in agriculture and plant biotechnology but also in pharmacology and beauty cosmetics.

Discovery of Cytokinins

The first experimental indication of the existence of CKs was reported by the intellectual aristocrat Gottlieb Haberlandt (1913), who noted that non-dividing potato parenchyma cells can be induced to divide in the presence of phloem sap from various plant species. He suggested that a soluble material occurring in phloem was responsible for the induction of cell division. In accordance with what was then the new concept of hormones as specific chemical signals coordinating activities and growth of different parts of the animal body (Starling 1905), Haberlandt assumed that the inducing factor is of hormonal nature. Actually, CKs do not meet the technical criteria of the definition of a

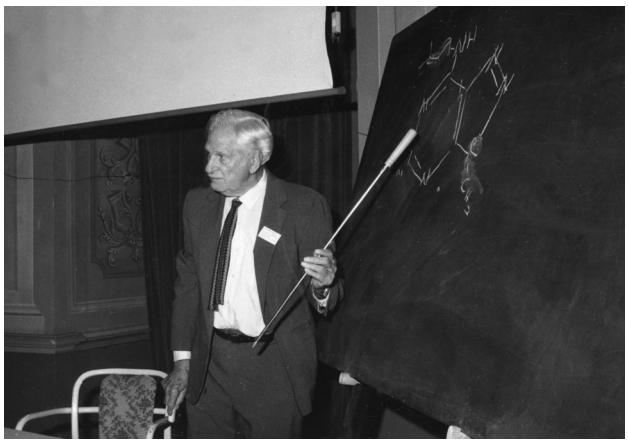

Fig. 2 Prof. Folke Skoog (Symposium Physiology and Biochemistry of Cytokinins in Plants, Liblice, Czech Republic 1990)

hormone, and the term "plant hormone" is used rather as a matter of convenience not only in this essay.

The active cell division factor was identified 42 years later in the laboratory of Prof Folke Skoog at the University of Wisconsin, Madison, USA. Skoog became interested in this problem during studies of the stimulatory effects of external chemical factors on the initiation and growth of callus and buds in tobacco stem cuttings (Fig. 2). It was found that addition of adenine to the culture medium reversibly counteracted the inhibiting effect of auxin on bud formation and also enhanced growth of callus in the presence of auxin (Skoog and Tsui 1948, 1951). This effect could be related to limited but detectable incorporation of radiolabeled adenine into CKs (Chen and Petschow 1978; Dickinson and others 1986). For a number of reasons, tobacco stem pith (consisting of parenchyma cells without vascular tissues), as well as the corresponding callus tissues, appeared to be much more uniform materials than stem segments for testing the effects of exogenous chemical factors on growth and differentiation of plant tissues. Experiments with tobacco stem pith tissues showed that auxin was capable of inducing mitosis unaccompanied by cytokinesis (Naylor and others 1954). Cell division was achieved by the addition of certain natural products such as coconut milk (introduced by van Overbeek and others 1941) to mineral medium containing auxin. Evidently these products contained bioactive factor(s) that in the presence of auxin were capable of maintaining continuous cell division. The stage for the hunt for the "elixir" of cell proliferation was set.

A substantial role in this chase was played by Carlos Miller, a new post-doctorate fellow who joined Skoog's laboratory after receiving his PhD at Ohio State University. He found that an active factor, which was present in some preparations of yeast extract, could be precipitated by silver nitrate, indicating that it might be a purine or

pyrimidine derivative. Considering the reported positive effects of adenine on bud formation and callus growth, the alternative that the growth factor could be a purine was preferred. Testing of various purine derivatives and potential sources of purine compounds showed that commercial preparations of herring sperm DNA, either old or artificially "aged" by autoclaving under acidic conditions, exhibited high cell division promoting activity. Following extraction of autoclaved DNA preparations with n-butanol at pH 6.8, evaporation of the butanol fraction, and passing the residual solution through the cation-exchanger Dowex 50 (H^+), the active compound was eluted by 1.5 N HCl. Entrapment of pooled active fractions on a similar column and elution with 1 N ammonium hydroxide provided the active compound in a crystalline form that was visible moving down the column. Its chemical characteristic and properties, including the empirical chemical formula and an equivalent molecular weight, were determined with the aid of Prof Frank Strong and associates from the Department of Biochemistry (Miller and others 1955a, b). Precisely, the first crystals of kinetin were isolated on December 16, 1954 (Amasino 2005). The paper reporting the isolation and chemical properties of the compound that was named kinetin with respect to its ability to promote cytokinesis was submitted to the *Journal of the American Chemical Society* in January 1955 (Miller and others 1955a). On the basis of the chemical properties and reactivity of the isolated compound with different reagents, Miller correctly proposed the chemical structure of kinetin as 6-furfurylaminopurine, which was confirmed by synthesis (Miller and others 1955b; for details see Skoog 1994 and Amasino 2005). Availability of kinetin facilitated further research on the functioning of CKs in the control of plant development. Demonstration of the dependence of the induction of shoot and root formation in cultured tobacco callus on kinetin/auxin concentration ratios in culture medium has been a good example (Skoog and Miller 1957). The importance of these findings for the advancement of plant biology and biotechnology is difficult to overestimate.

Discovery of kinetin as the first compound assigned to the class of plant growth regulators called now CKs represents a narrative story of targeted search for an anticipated factor involved in the control of fundamental developmental processes in plants. It included a meaningful leitmotif, establishment of a sensitive and specific bioassay, the search for a rich source of active compound, and application of effective separation and purification methods. It also required competent actors, who were capable of laying out a prominent objective and of skilled experimentation as well as a tenacious efforts to reach the target. Of course, good luck has always been an important component of success.

Availability of kinetin allowed preparation of completely defined culture media for continuous cultivation of plant tissues under in vitro conditions. To optimize the system, the mineral and organic components of the medium of White (White 1943), commonly used at that time, were fundamentally modified. The revised medium (Murashige and Skoog 1962 and Linsmaier and Skoog 1965) was designated for use in bioassays of organic growth-promoting compounds. However, it has been widely used not only for testing biological activity of CKs but also in plant biotechnology as indicated by the enormously high citation records, according to my knowledge, the highest in plant biology (39,334 and 2958, respectively, to June 5th, 2015 in WOS database).

Search for Natural Cytokinin(s)

Cytokinins Bearing N^6-Isopentenyl Side Chain

Kinetin represented a powerful tool for investigation of structure–activity relations and the biological functions of CKs. However, it was not a natural plant hormone but a secondary oxidative product of DNA (Hall and De Ropp 1955; Barciszewski and others 1997). The search for natural cell division stimulant(s) was already under way in the laboratory of Prof F. C. Steward at Cornell University prior to the discovery of kinetin. In fact, the unwillingness of Steward to provide information about the procedure employed in his laboratory for purification of coconut milk prior to its use as a component of culture media evoked Skoog to focus on a search for the "cell division factor" (Skoog, personal communication; Amasino 2005). According to Letham (1999), at least eight laboratories were engaged in isolation of the active compound(s) by 1960. Investigators faced serious problems—the active compound(s) occur(s) in minute quantities among the bulk of other structurally similar substances in natural products, including inactive purines. The availability of a huge amount of coconut milk, collected from coconut trees knocked down by a hurricane in Florida provided Shantz and Steward (1955) with enough material for separation and identification of a cell division factor. From this source, they isolated *N*,*N*′-diphenylurea, which promoted cell division in phloem parenchyma of carrot roots, indicating that it could be a natural CK. However, there was some doubt about potential contamination of the preparation during processing, which occurred in a commercial facility previously used for the synthesis of urea herbicides (Jacobs 1979). Actually, the presence of *N*,*N*′-diphenylurea in coconut milk has not been confirmed to date. Nevertheless, a number of synthetic aromatic derivatives of urea exhibit CK activity much higher than *N*,*N*′-diphenylurea itself (Bruce and Zwar 1966). Some of them, like

thidiazuron, are widely used in plant biotechnology (Gupta and Bhargava 2001; Matand and Prakash 2007). On the contrary, kinetin and its riboside were unambiguously identified in coconut milk (Kobayashi and others 1995; Ge and others 2005) and some other materials (Barciszewski and others 1996, 1997). However, accumulating knowledge about the biosynthesis, metabolism, and signaling of natural CKs showed that neither aromatic ureas nor kinetin are functional natural plant hormones.

However, there was an early indication that at least some natural CK was a purine derivative. Carlos Miller, who became a professor at the University of Indiana, turned his attention to immature maize kernels, which appeared to be a rich source of a cell division factor and demonstrated that it is a purine-like compound closely related to kinetin (Miller 1961).

Beginning in 1959, research seemingly unrelated to CKs proceeded at another site on the globe in the Fruit Research Division of the Department of Scientific and Industrial Research in New Zealand. This research was carried out in a temporary laboratory called "The barn", which was built over a farm-machinery storage shed at Mount Albert. The research was focused on prevention of internal breakdown of apple fruit during cold storage (Letham 1999). Among numerous investigated factors, the size of cells forming the mid-cortex of apple fruits positively correlated with the high incidence of internal fruit degradation (Martin and others 1954). The team reasonably deduced that stimulation of cell division may reduce the cell size and thus prevent the storage disorder. For testing the activity of compounds stimulating cell division in young fruits, an in vitro culture of apple fruit tissue was established. Similarly to cultures of other plant species, it required addition of extracts from coconut milk or young maize kernels as a source of the cell division stimulant (Letham 1958). Extracts from plum and apple fruitlets were found to be excellent sources of this activity (Letham and Bollard 1961; Letham 1963a). However, extraction and fractionation of 30 kg of young plums did not yield amounts sufficient for identification of the active compound, which appeared to be a purine derivative (Letham 1963b).

The New Zealand investigators switched to the extraction of immature maize kernels as a rich source of purine-like cell division factor(s) closely related to kinetin. In April 1963, extraction and fractionation of 60 kg of sweet maize kernels yielded 0.7 mg of crystalline pure compound identical to the plum factor and stimulating cell division in the carrot tissue culture assay at a concentration as low as 0.1 μg l^{-1}.The new natural plant hormone was named zeatin after the source plant species, *Zea mays* (Letham 1963c, 1966). Determination of the structure of zeatin involved mass spectrometry, used for the first time in plant hormone research. The assigned structure of zeatin, 6-(4-hydroxy-3-methylbut-*trans*-2-enylamino)purine (*trans*Z), (Letham and others 1964), was unambiguously confirmed by synthesis (Shaw and others 1966; Cebalo and Letham 1967).

Miller isolated independently the cell division stimulant from the same plant source in a very pure form, which, as shown later, was identical to *trans*Z (Miller 1965; Letham and Miller 1965). Interestingly, *trans*Z riboside was subsequently unequivocally identified also in coconut milk (Letham 1974), that is, in the plant material where the original search of Shantz and Steward (1955) failed.

Isolation and determination of the chemical structure of *trans*Z has represented an inspiring story of how applied research can initiate investigations of fundamental factors controlling plant development regardless of limited funding and experimental conditions. According to Letham "In 1963 we were uncertain if zeatin is really a plant hormone, or just a compound unique to *Zea mays*. In those days the work was difficult; HPLC did not exist… …to determine the structure of zeatin 0.6 mg of crystalline compound, recrystallized to constant melting point, gave only one mass spectrum. Today zeatin is everywhere, even in the local pharmacy in a skin cream to cure my wrinkles" (personal communication, 2014) (Fig. 3).

Cytokinins with an Aromatic Ring in the N^6 Side Chain

The first analogue of kinetin bearing an aromatic side chain, N^6-benzyladenine (BA), was synthetized by Prof F.M. Strong and his associate F.S. Okimura within a few days after the structure of kinetin was determined (see Armstrong 2002). The first natural aromatic CK bearing the phenyl ring hydroxylated in the *ortho* position was unambiguously identified in poplar leaves by Horgan and others

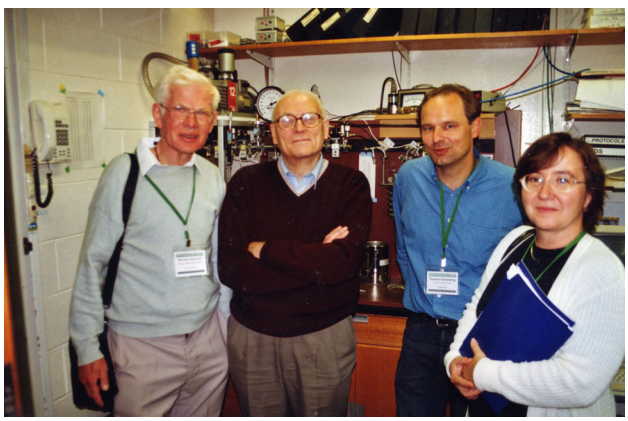

Fig. 3 Prof. DS Letham in his former laboratory in Canberra, Australia, 2004 (from *left* M. Kaminek, Prof. DS Letham, Prof. T. Schmülling, R.Vaňková)

(1973) and similar *meta* hydroxyl derivative was found in the same plant material by Strnad and others (1997). The latter was found to exhibit the same CK activity as *trans*Z in four different bioassays (Kamínek and others 1987). Thanks to the development of sensitive analytical methods, Strnad and his associates detected aromatic CKs hydroxylated on the N^6 side-chain phenyl ring in a number of plant species and named this group of CKs "topolins" according to the Czech name of poplar, from the leaves of which the first compound of this type was isolated (see Strnad 1997 for review).

Historical Similarities of the Search for Natural Auxin and Cytokinin(s)

There are interesting parallels in the identification of natural auxin and CK. Structures of the first two isolated auxins (auxin A and auxin B) published by Kögl and others (1933) did not contain an indole bicyclic nucleus, and the existence of the corresponding natural compounds has not been confirmed. The structure of the natural auxin, indole-3-acetic acid (IAA, **hetero**auxin), was determined in the same laboratory one year later (Kögl and others 1934). Similarly, N,N'diphenylurea was identified as the first natural CK in extracts of coconut milk (Shantz and Steward 1955); but again its natural occurrence has not been proven. Moreover, the structure of both IAA and kinetin was determined following analysis of materials of animal origin, that is, human urine and herring sperm DNA, respectively. Both CKs and auxins are essential for cooperative control of basic biological processes in plants, that is, cell division and differentiation (Skoog and Miller 1957; Bielach and others 2012; Marhavý and others 2014).

Cytokinins in Transfer RNA

Cytokinins as Components of Certain tRNAs

Shortly after sequencing the first tRNA, that is, $tRNA^{Ala}$ (Holley and others 1965), a modified nucleoside, N^6-(Δ^2-isopentenyl)adenosine (iPR) was identified next to the 3′-end of the anticodon in two yeast $tRNAs^{Ser}$ (Zachau and others 1966). Subsequently, iPR was also found in plant soluble RNA (Hall and others 1967; Letham and Ralph 1967). This nucleoside and the corresponding base (iP) were already known to be highly active CKs (Hamzi and Skoog 1964; Hall and others 1966; see also Skoog 1994).The presence of CK moieties in certain tRNAs evoked speculation that the biological action of CKs might possibly be the result of CKs serving as precursors of the modified substituent of some tRNAs. Indications that a fraction of radioactivity derived from exogenously applied [^{14}C] BA to tobacco cultures was found in soluble RNA, principally tRNA, (Fox 1965) supported such suggestions. However, it soon became evident that the mechanism of CK action is not based on CK incorporation into tRNAs. Over 90 % of radioactivity recovered from RNA preparations from tobacco callus tissue grown on media containing either [^{14}C]BA or [^{14}C] BA riboside was associated with rRNA. Moreover, the amount of incorporated [^{14}C] BA was too low to be physiologically significant (Armstrong and others 1976). Feeding of tissues with labeled mevalonate showed that the side chain of CK moieties in tRNA of *Lactobacillus* (Peterkofsky 1968), yeast, and rat liver (Fittler and others 1968) were derived from mevalonate. Moreover, their formations in cultured tobacco pith tissue plant tRNA was not suppressed by exogenous CK (Chen and Hall 1969).

Extensive analysis of tRNAs from different sources proved that moieties of isoprenoid CKs occur as structural parts of certain tRNA species of all organisms from eubacteria (but not in archaebacteria) to humans (for review see Persson and others 1994). Located at position 37 adjacent to the 3′end of the anticodon loop of only those tRNAs recognizing codons starting with U, as reported for *Escherichia coli*, yeast, wheat germ and bean seedlings (Armstrong and others 1969; Struxness and others 1979; Edwards and Armstrong 1981), the CK moieties of tRNA facilitate codon-anticodon interaction and enhance the efficiency of tRNA in translation (Gefter and Russell 1969) by stabilizing mRNA-tRNA pairing and by the maintenance of the reading frame (Czerwoniec and others 2009; Agris and others 2007). Although in *E. coli* CK activity is associated with almost all tRNA species and subspecies of the U-coding group (Armstrong and others 1969), the presence of CK moieties was found to be much more restricted in plant tRNAs. In wheat germ and etiolated *Phasoleus vulgaris* seedlings, the CK activity in tRNA species was associated only with $tRNA^{Ser}$ and one minor $tRNA^{Leu}$ (Struxness and others 1979; Edwards and Armstrong 1981). An instructive example of this evolutional restriction is the replacement of the isoprenoid moiety occurring in bacterial $tRNA^{Phe}$ with wybutosine (base Y) in $tRNA^{Phe}$ of eukaryotes, including higher plants (Young and Bandarian 2013). Wybutosine is a heavily modified guanosine derivative which does not have CK activity (Barciszewska and others 1981) but supports the function of tRNA in translation (Carlson and others 1999). Moreover, plant tRNAs contain moieties of *cis*Z which exhibits much lower CK activity compared to the *trans* isomer (Schmitz and others 1972; Kamínek and others 1979).

Relationships of tRNA and Free Cytokinins

Comparison of the occurrence and functions of free and tRNA-bound CKs in different organisms has demonstrated that their appearance in tRNA is not accidental. It has been proposed that the first hormone-like structures that appeared in living cells were iPR and/or its derivatives, which were released from bacterial tRNA. Their functioning as plant hormones only became biologically significant with the evolution of multicellular plants (Kamínek 1974). To acquire hormonal functions, precise regulation of the concentration of free hormone is indispensable for modulating plant development. This has required the development of efficient control of hormone levels independent of tRNA turnover. Powerful mechanisms preventing interference of the CKs of tRNA origin with CKs involved in hormonal regulations were developed during plant evolution (Kamínek 1982). As indicated above these mechanisms include (1) a reduction of the number of tRNA species bearing CK moieties in plants compared to other organisms, (2) preferential modifications of plant tRNAs with *cis*Z riboside compared to iPR moieties and (3) adaptation of CK recognition systems to respond more readily to *trans*Z compared to the corresponding *cis* isomer. This allowed differentiation of zeatin functions in tRNA translation (*cis*Z) from hormonal regulations (*trans*Z) (Kamínek 1974, 1975, 1982). Nevertheless, *cis*Z and its riboside retain low CK activity, and there are indications that *cis*Z-type CKs participate in sustaining some basic physiological processes in plants exposed to environmental conditions unfavorable for growth. Restoration of plant growth is associated with an increase in the levels of the *trans*Z-type of CKs (Gajdošová and others 2011).

Cytokinin Biosynthesis

Elucidation of the pathways of CK biosynthesis represents an exciting story in plant hormone research. There was a long gap between the chemical identification of CKs and shedding light on their biosynthetic pathways. It even evoked suggestions that CKs are not produced by plants but exclusively by plant microbial symbionts (Holland 1997). If this was the case, CKs could not be considered as plant hormones (Romanov 2011). However, the presence of CK moieties in certain tRNA species of a broad spectrum of organisms (including plants), production of free CKs by certain plant-associated microorganisms, and the ability of some phytopathogenic bacteria to transform cells of the host to synthesize CKs, as well as the production of CKs by untransformed plants, offered numerous experimental materials and approaches to solve this problem. Nevertheless, it took 47 and 37 years, respectively, since kinetin and the first plant natural CK, *trans*Z, were identified to decipher the biosynthetic pathway of isoprenoid CKs in higher plants.

tRNA as a Potential Source of Free Cytokinins

Thanks to the fast progress of molecular biology in the 1960s and 1970s of the last century, it has been evident that, similarly to other modifications of RNA, the prenylation of tRNA proceeds at the polynucleotide level (for review see Murai 1994). As Chen and Hall already reported in 1969, the radioactivity from radiolabeled mevalonic acid supplied to cultured tobacco pith was significantly incorporated into the iPR tRNA component. This, together with the finding that modified bases including CKs can be released during tRNA degradation (McLennan 1975), suggested that tRNAs can be a source of free CKs in plant cells. Actually, six different compounds exhibiting different CK activities have been detected in plant tRNA hydrolysates: *cis*ZR, *trans*ZR and iPR and their 2-methylthio derivatives (see Taller 1994). As calculated by Barnes and others (1980), up to 40 % of free CKs produced by a potato cell culture could be of tRNA origin, but this still indicated that the predominant amount of CKs should be synthesized by an another pathway. In addition to tRNA, isopentenylated mRNA and prenylated oligonucleotides have also been considered as potential sources of free CKs (Holtz and Klämbt 1978). However, this potential source of free CKs has not been further investigated. Exhausting arguments suggesting that free CKs were predominantly synthesized by an alternative pathway were summarized by Letham and Palni (1983). However, CKs of tRNA origin may represent a minor source of free CKs, maintaining a low level CK background. Such a system should react readily to changes in the CK supply via direct CK biosynthetic pathway(s) responding to developmental and environmental signals.

Direct tRNA-Independent Cytokinin Biosynthesis

A direct *de novo* CK biosynthetic pathway in plants was predicted on the basis of the numerous experimental indications described above. Taya and others (1978) were the first to report biosynthesis of free CK in vitro. They demonstrated that cell-free extracts of the cellular slime mold, *Dictyostelium discoideum,* catalyzed the attachment of the isopentenyl moiety from dimethylallyl diphosphate (DMAPP) to AMP but not to ADP or ATP. The slime mold cells contain a large amount of free iP, which is further metabolically converted to discadenine, a spore germination inhibitor that also exhibited CK activity in the tobacco bioassay (Nomura and others 1977). The DMAPP:AMP isopentenyltransferase (IPT) activity was detected in

plants, and the enzyme was partially purified from higher plant cells, including CK autotrophic tobacco tissue (Chen and Melitz 1979) and cell cultures (Nishinari and Syono 1980). Its activity was subsequently detected in extracts of immature *Zea mays* kernels (Blackwell and Horgan 1994). The enzyme was never purified to full homogeneity, either because of its low content in plant cells and/or its extreme instability. It is also possible that some (if not most) activity escaped detection due to the use of AMP as the acceptor of DAMPP in the enzyme assays. According to our present knowledge, the preferred substrates of plant adenylate-IPTs are ADP and ATP (Kakimoto 2001, 2003a, b).

Identification of Cytokinin Biosynthetic Genes Involved in Crown Gall Disease

In spite of intensive efforts, the isolation and precise characterization of IPT(s) from plants and cloning of the corresponding gene(s) was not successful. Much more productive were investigations of the initiation and retention of auxin- and CK-autonomous growth of tumors (crown gall) caused by the plant pathogen *Agrobacterium tumefaciens* (formerly *Phytomonas tumefaciens*). Crown gall disease, as well as isolation of the causal bacterial pathogen, was, according to Kado (2014), described by Fridiano Cavaraevokes already in 1867. However, the more recent paper of Smith and Townsend (1907) who named the causal organism *Agrobacterium tumefaciens* has become the most frequently quoted in this respect (for review see Kado 2014).

The fascinating story of the elucidation of the functioning of auxin and CKs in tumor initiation and successive plant genetic transformation began with the finding that auxin stimulates growth of attenuated crown gall tumors (Went and Thimann 1937) and that tumors are sources of auxin (Link and Eggers 1941). The subsequent discovery that secondary tumors arising at distant sites from primary ones are often free of bacteria and retain tumor character when grown on media lacking growth-promoting substances (White and Braun 1941, 1942; Braun and White 1943) indicated that the host cells become permanently altered by the pathogen. Two distinct phases of tumor formation were recognized, that is, the initial conditioning phase where cells of the host are stimulated to divide but remain dependent on a supply of growth-promoting substances and the second induction phase resulting in formation of tumors capable of indefinite cell division (Braun 1947; Braun and Laskaris 1942). It was obvious that conversion of hormone-dependent tissues to autonomous ones can be caused either by permanent activation of the CK and auxin biosynthetic pathways of the host plant or by transfer of the corresponding biosynthetic capacity from the pathogen to the host. Association of the transformation with the action of a hypothetical "tumor inducing principle" (TIP), which was carried by the pathogen and could be inactivated by mild heat treatment at different stages of the transformation process (Braun and Mandale 1948), supported the second alternative. However, it was too early to consider a transfer of genetic information from the pathogen to the host—techniques of DNA cloning were discovered 24 years later. Nevertheless, Braun's consideration of the activation of CK and auxin biosynthesis during the transition process was correct. It is fascinating how Braun's brilliant deductions, arising from limited but well-founded experimental backgrounds reached and in certain aspects even exceeded the limits of scientific knowledge available in his time. Following the discovery of kinetin, Braun (1958) suggested that "It is possible for a cell to acquire the capacity for autonomous growth as a result of the permanent activation of a series of growth-substance-synthesizing systems, the products of which are concerned specifically with growth accompanied by cell division". It is amazing how Braun's conclusions anticipated the two-step process of host plant transformation involving the virulence of infection and subsequent genetics transformation of susceptible plant cells.

Consideration that permanent hormonal autonomy of crown gall tumors is caused by genetic transformation was strengthened by identification of opines, unusual arginine conjugates such as nopaline and octopine, which are excreted by tumor cells induced by different *A. tumefaciens* strains that are able to metabolize the respective opines as carbon and nitrogen sources (Ménagé and Morel 1964). Moreover, Zaenen and others (1974) discovered the presence of a large circular plasmid that was specific to the virulent bacterial strains and proposed that it could serve as a TIP carrier. Subsequent hybridization of radiolabeled fragments of plasmid DNA with tobacco tumor DNA proved that only part of the plasmid DNA (T-DNA) is transferred to the tumor DNA (Chilton and others 1977) and integrated into the plant nuclear genome (Yadav and others 1980; Zambryski and others 1980). Using transposon insertions, the T-DNA loci affecting tumor morphology were recognized. Mutants inciting rooty (*tmr*) and shooty (*tms*) tumors indicated that specific T-DNA loci influence phytohormone levels (Garfinkel and others 1981). Indeed, in accordance with the dependence of root and shoot formation on auxin/CK concentration ratios in tobacco stem pith tissue (Skoog and Miller1957), extensive shoot formation in *tms* tumors was found to be accompanied by high CK/auxin ratio while the opposite ratio was recorded in root forming *tmr* tumors (Akiyoshi and others 1983; see Morris 1986 for review). Two research groups reported almost simultaneously that the *tmr* locus is responsible for CK overproduction in *tmr* crown gall

tumors by encoding a DMAPP:AMP IPT that catalyzes the first step in CK biosynthesis, that is, formation of isopentenyladenosine 5′-phosphate (iPRMP) from DAMPP and 5′AMP (Akiyoshi and others 1984; Barry and others 1984). Taking into account that this locus encodes IPT, the second group proposed to rename it *IPT*. Similarly to the IPT from *Dictyostelium discoideum,* the *tmr* enzyme did not isopentenylate ADP and ATP (Morris and others 1993). In addition to *IPT*, another gene of very similar sequence homology responsible for the biosynthesis and secretion of *trans*Z (*TZS*) was found located outside the T-DNA but close to the region associated with virulence functions in the *A. tumefaciens* nopaline strain (Akiyoshi and others 1985; Beaty and others 1986).

One more discovery was achieved in this line of research for CK biosynthetic pathways. A detailed study of *A. thaliana* plants transformed with *A. tumefaciens IPT* showed that high accumulation of *trans*ZRMP compared to iPMP is caused by operation of a new iPMP-independent biosynthetic pathway. Using the terpenoid 1-hydroxy-2-methyl-*E*-butenyl 4-diphosphate (HMBDP) as a side-chain donor allows bypassing the conventional CK biosynthesis pathway without the requirement for further iPR metabolic side-chain hydroxylation (Astot and others 2000). Nevertheless, DAMPP is the preferred site-chain donor in non-transformed plants (Sakakibara and others 2005).

Investigation of the mechanisms of plant transformation by *A. tumefaciens,* including the transfer of genes encoding CK and auxin biosynthesis, provided valuable knowledge, which has been exploited in the search for IPTs catalyzing CK biosynthesis in higher plants. This research attracted unusual attention and funding, thanks to its potential for providing a vehicle for genetic transformation of plants and the resulting applications in plant biotechnology.

Identification of Cytokinin Biosynthetic Enzymes and Genes in Higher Plants

Complete sequencing of the *Arabidopsis thaliana* genome was achieved in 2000. Thus, the first complete genomic sequence for a flowering plant provided a powerful tool for identification of genes encoding CK biosynthetic enzymes. Just the next year, the genomic sequences encoding a small family of IPTs were independently and almost simultaneously published by Takei and others (2001) and Kakimoto (2001), both from Japan. Screening the genomic sequence of *Arabidopsis thaliana* in silico against the amino acid sequence of *Agrobacterium TMR,* Takei and others (2001) identified eight IPTs encoded by cDNAs with significant homology to *TMR*. The genes for the IPTs, designated as *AtIPT1* to *AtIPT8,* were distributed on all five chromosomes. When expressed *in E. coli* under an inducible promoter, all *AtIPT*s but *AtIPT2* caused secretion of CKs, predominantly iP, into the culture medium, indicating that AtIPT2 catalyzes prenylation of tRNA. Analysis of the reaction catalyzed by purified recombinant AtIPT1 confirmed formation of free iPMP from DMAPP and AMP.

At the same time Kakimoto (2001) searched the *Arabidopsis* genomic sequence for genes that could code for IPTs. However, a standard BLAST search of the database using bacterial CK biosynthetic DMAPP:AMP IPTs as a query proved plant sequences with only weak resemblances to the bacterial genes. Involvement of amino acid residues that were common to both bacterial DMAPP:AMP and DMAPP:tRNA-IPTs, that is, IPTs recognizing different substrates, allowed the Patmatch program to be used to identify also IPTs for unknown substrates and led to the recognition of nine putative genes for different IPTs (*AtIPT1 to AtIPT9*). Examination of crude extracts of *E. coli* expressing AtIPT4 showed that the IPT activity was lost after purification on an affinity column, possibly due to the removal of *E. coli*-derived factors, namely adenosine kinase which catalyzes phosphorylation of AMP to ADP. Indeed, ATP and ADP but not AMP acted as substrates of the purified AtIPT4. Correspondingly, AtIPT4 represents a DMAPP:ATP/ADP IPT, while as reported by Morris and others (1993), TZS from *A. tumefaciens* uses AMP as a sole acceptor of isopentenyl moiety. Moreover, overexpression of *AtIPT4* in tobacco calli resulted in the regeneration of shoots in a manner resembling the effects of a high CK/auxin concentration ratio in culture medium (Skoog and Miller 1957).

Thanks to the contact of Kakimoto with Sakakibara, both researchers reached an agreement to use the same nomenclature for the *Arabidopsis* genes (*AtIPT1-9*) (see Kakimoto 2003a for details). This, as well as the simultaneous submission of their manuscripts for publication, has been a nice example of the mutual respect of two scientists for the research of each other and for their corresponding priorities. Coincidentally, both Sakakibara and Kakimoto presented the identification of CK biosynthetic genes in *A. thaliàna* at the *U.S.A.—Czech Republic International Workshop on the Plant Hormone Cytokinin* and the *17th Integrational Conference on Plant Growth Substances,* which were held in Prague and Brno (Czech Republic) in June and July, respectively, in 2001. These probably were the first presentations of this important discovery outside Japan. It received unusual and well-deserved attention of the scientific audience.

As reported afterwards by Takei and others (2004), free iPR generated via the IPT pathway, as well as the corresponding base, can be further stereospecifically hydroxylated to *trans*Z forms by cytochrome P450 monooxygenases CYP735A1 and CYP735A2. Thus, the complete biosynthetic pathway of the most widely occurring and bioactive CKs in untransformed plants was elucidated.

Nevertheless, the only biosynthetic pathway so far known for *cis*-zeatin is the indirect one via tRNA (Miyawaki and others 2006). However, the restricted isoprenylation of plant tRNAs with respect to other organisms (Struxness and others 1979; Edwards and Armstrong 1981) compared with high levels of *cis*-zeatins in a great number of plant species across the plant kingdom (Gajdošová and others 2011) should encourage a search for a separate biosynthetic pathway of these "bifunctional" CKs.

Existence of some other as yet unknown biosynthetic pathway(s) of adenylate CK(s) cannot be excluded. Interesting information in this respect was provided by recent analysis of free and tRNA-bound CKs in the moss *Physcomitrella patents*. The genome of this early land plant encodes only homologues of tRNA-IPTs but no adenylate-IPTs. Targeted knockout of the gene encoding IPT1, which was almost exclusively responsible for tRNA prenylation, reduced the levels of free *cis*Z whereas the contents of bioactive iP- and *trans*Z-type CKs were increased indicating that their biosynthesis may proceed via another tRNA-independent pathway (Lindner and others 2014).

What remains a mystery is the biosynthesis of aromatic CKs. Forty two years after the first aromatic CK (Horgan and others 1973), there is no indication of how CKs bearing side chains with an aromatic ring are synthesized *in planta*.

Metabolisms of Cytokinins

Levels of bioactive CKs in plant cells are, in addition to CK biosynthesis, regulated by metabolic conversions of CKs to (1) compounds exhibiting modified CK activity, (2) storage forms of CKs, and (3) inactive degradation products. Availability of radiolabeled CKs allowed pioneering studies of the metabolism of exogenously applied CKs, followed by identification of the corresponding metabolic enzymes and genes (for reviews see Letham and Palni 1983; Mok and Mok 2001; Galuszka and others 2008; Frébort and others 2011).

Metabolic Conversions of Exogenous Cytokinins

Already, the first study of the metabolic fate of labeled CK, [8-^{14}C]*trans*Z, applied to a plant, that is, excised bean embryonic axes, indicated incorporation of radioactivity into ZR, ZRMP, and dihydrozeatin (DHZ) (Sondheimer and Tzou 1971). Feeding of de-rooted radish seedlings with [^{3}H]*trans*Z via the transpiration stream demonstrated that the major metabolite in the aerial organs is a glucoside termed raphanatin (Parker and others 1972), which was later unequivocally identified as 7-β-D-glucopyranosylzeatin (Parker and Letham 1973). An analogous application of radiolabeled *trans*Z to excised maize roots resulted in its conversion to *trans*Z 9-glucoside and *trans*Z 7 glucoside as the major and minor metabolites, respectively (Parker and others 1973). Several other labeled metabolites, including ZRMP, AMP, adenine, and adenosine, were detected in radish and/or maize seedlings by chromatographic methods. Following application of [^{3}H]*trans*Z to de-rooted lupin seedlings, two more metabolites were unequivocally identified, *trans*Z 9-alanine (named lupinic acid) and *trans*Z *O*-β-D-glucoside (Parker and others 1978). These pioneering experiments clearly indicated that the metabolic control of levels of *trans*Z represents a complex system, involving modifications of the adenine moiety and side chain as well as CK degradation by side chain removal.

Cytokinin Degradation by Cytokinin Oxidase/Dehydrogenase

Biosynthesis and degradation represent the two major metabolic processes controlling the levels of bioactive isoprenoid CKs in plant cells. Enzymatic activity catalyzing conversion of iPR to adenosine was firstly detected in crude extract of tobacco tissue by Pačes and others (1971). Subsequently, a partially purified enzyme from *Zea mays* kernels was shown to degrade natural iPR via an unstable intermediate. The reaction required oxygen, and assuming that the cleavage involved an oxidase-type mechanism, the enzyme was designated as cytokinin oxidase (CKX) (Whitty and Hall 1974). The products of iPR cleavage were identified as adenosine and the corresponding aldehyde of the side chain (Brownlee and others 1975). CKX activity has been recorded in many plant species and reported to play an apparently crucial role in the control of CK levels *in planta* (see Armstrong 1994; Mok and Mok 2001; Hare and Van Staden 1994). Importantly, the CKX degradative activity in tobacco cell suspension cultures was found to be under the control of CKs themselves (Terrine and Laloule 1980), that is, being induced by the substrate as reported also for *Phaseolus* callus tissues (Chatfield and Armstrong 1986; Kamínek and Armstrong 1990) and for tobacco calli and plants expressing IPT gene (Motyka and others 1996). At least some isoforms of CKX were found to be glycosylated (Chatfield and Armstrong 1988; Kamínek and Armstrong 1990), and upregulation of CKX by CKs in tobacco cell suspensions, calli and leaves was associated predominantly with the glycosylated form of the enzyme (Motyka and others 2003).

Detailed characterization of the biochemical properties and kinetic parameters of CKX was initially restrained by the limited availability of CKX of sufficient purity. CKX genes were cloned simultaneously in two laboratories using partial amino acid sequences of the enzyme purified from immature maize kernels, which represent a rich source of

CKX activity (Dietrich and others 1995). Morris and others (1999) purified the CKX1 to homogeneity and used selected tryptic peptides to design primers for isolation of corresponding fragments of CKX gene and subsequent isolation of full-length CKX1 gene from maize genomic library. Houba-Hérin and others (1999) exploited the ability of phenylureas to inhibit CKX for photolabelling and spotting of a glycosylated CKX, determination of the peptide sequence of the labeled protein, and cDNA cloning. Interestingly, the two clones isolated by the two research teams differed in only seven nucleotides (Mok and Mok 2001).

Expression of an intronless ckx1 from maize in *Pichia pastoris* allowed production of large amounts of enzyme and subsequent kinetics and cofactor analysis of the recombinant CKX. The enzyme was found to be a flavoprotein consisting of three exons separated by two introns, predicted signal peptide and eight possible N-glycosylation sites. Seven homologs, located on three different chromosomes, were identified in *Arabidopsis* (Bilyeu and others 2001).

Using a novel spectrophotometric assay (Morris and others 1999), iP, *trans*Z, and iPR were identified as good substrates for both native and recombinant CKXs. *cis*Z and *trans*ZR were degraded only by the recombinant CKX, whereas dihydrozeatin (DHZ), kinetin and BAP were not degraded by either enzyme (Bilyeu and others 2001). The spectrophotometric assay is based on ability of the enzyme to use an electron acceptor other than oxygen. It displays a dual catalytic mode for degradation of CKs: a low rate and low substrate specificity reaction, using oxygen as an electron acceptor, and a high activity and strict specificity for isoprenoid CKs with some specific electron acceptors, for example, quinones (Frébortová and others 2004). These properties of the enzyme correspond to the most frequently used denomination of the enzyme as CK oxidase/dehydrogenase (CKX).

Cytokinin Signaling

Looking for Cytokinin-Binding Sites…

The search for CK binding sites in the early 1970s of the last century can be designated as the very beginning of experimental search for recognition of a CK message. Regardless of the potential existence of signaling pathway(s) it was clear that a specific resolution of CK signal is a prerequisite of CK action. The search started with recognition of equilibrium-type binding of kinetin and BA to purified chinese-cabbage ribosomes (Berridge and others 1970). Subsequently a number of CK binding proteins (CBPs) were identified in different plant materials (for review see Brinegar 1994). High amounts of a CBP that preferentially bound aromatic CKs were found in wheat germ (Erion and Fox 1981). However, this protein functioned in sequestering or temporal immobilization of aromatic CKs (Brinegar and Fox 1987) rather than in hormonal regulations (Kamínek and others 2003). In spite of the groan of Firn (1987) that "There are too many CBPs but not enough receptors" the research on CBPs contributed to the development and adaptation of sophisticated techniques and selecting criteria which have been helpful for the purification and characterization of true receptors.

Perception and Transduction of the Cytokinin Signal

A turning point in deciphering CK signaling in plants was the discovery that a novel ETR1 protein responding to ethylene in *Arabidopsis* plants is similar to that of the prokaryotic two-component signal system which bacteria use to sense and respond to external stimuli (Chang and others 1993). It appeared that a modified two-component signaling system also operates in plants (for review see Kakimoto 2003b). CKs are recognized by receptor hybrid histidine kinases (HKs), which are autophosphorylated in response to CK binding. The high-energy phosphoryl group is then transferred by sequential phosphorylation of histidine phosphotransfer proteins to phosphorylate the second component of the signal system, B-type transcription factors, that ultimately regulate the downstream signaling events. As reported by Kakimoto (1996), overexpression of a sensor kinase homolog CKI1 allowed CK-independent growth of cultured *Arabidopsis* tissues. Moreover, it was the first HK implicated in the perception of CKs. However, it does not contain a CK binding CHASE domain and does not bind CK in vitro (Yamada and others 2001). Subsequently, the first authentic CK receptor was identified by screening of *Arabidopsis* mutants for altered CK sensitivity. The mutated *CYTOKININ RESISTANT1* (*CRE1*), referred to as *AHK4,* was found to encode a membrane-located sensor HK which can be activated by CKs (Inoue and others 2001). In addition to CRE1/AHK4, two more AHK sensors (AHK2 and AHK3), were identified in *Arabidopsis* (for reviews see Kakimoto 2003b; Ferreira and Kieber 2005). There are convincing indications that CKI1 can activate the two-component phosphorelay in *Arabidopsis* via proteins involved in CK signaling (AHK2, AHK3) required for proliferation and maintenance of procambial and vascular stem cells (Hejátko and others 2009).

Availability of CK receptors fulfilled the dream of researchers to probe the "real" activity of different CKs by examining their ability to activate different CK receptors in heterologous systems or their affinity to receptors located on purified plant membranes, that is, in systems more or less free of interfering metabolic modifications of the

ligands. Heterologous functional expression of the CK receptors AHK4 or AHK3 in *E. coli,* coupled to an *E.coli* signaling pathway leading to the activation of an associated reporter gene for β-galactosidase, allowed rapid testing. Using this assay, Spíchal and others (2004) found that both investigated receptors CRE1/AHK4 and AKH3 recognize preferentially CK bases (iP, *trans*Z) compared to the corresponding ribosides and ribotides. Similarly, Yonekura-Sakakibara and others (2004) reported higher responses of CK free bases than corresponding nucleosides to the maize ZmHK1 and ZmHK3 receptors in the *E. coli* recombinant assay system.

A recently published alternative assay that proceeds in a more natural environment, that is, within plant membranes bearing the receptors (Lomin and others 2015), allowed testing also *Arabidopsis* receptor AHK2 which could be hardly expressed in *E. coli*. Comparison of the main CK binding characteristics of four *Arabidopsis* and one maize receptor showed a strongly reduced response of all tested receptors to CK 9-ribosides indicating that ribosides are rather transport forms of CKs with weak or no CK activity. Interestingly, this corresponds to the far-back finding of Laloue and Pethe (1982) who, using tobacco suspension cultures at low initial cell densities, proposed that conversion of CK ribosides to the corresponding bases is necessary for expression of their stimulatory effect on cell division. Interestingly, *Arabidopsis* receptors responded rather weakly to *cis*Z as compared to *trans*Z (Spíchal and others 2004; Lomin and others 2011) but all maize receptors (ZmHK1, ZmHK2 and ZMHK3a) remarkably responded to *cis*Z (Yonekura-Sakakibara and others 2004; Lomin and others 2011, 2015).This contrasts with the low activity of *cis*Z in various bioassays (Gajdošová and others 2011) and further fuels curiosity concerning the functioning of *cis*Z in the control of plant growth and development. Moreover, the potential association of the CK maize receptor ZmHK1 with the endoplasmic reticulum (Lomin and others 2011) has opened another line of research regarding CK receptors, their potential intracellular trafficking and pH sensing (Lomin and others 2015).

Origin and Evolution of Cytokinin Signaling

Transition of unicellular charophyte green aquatic algae to multicellular terrestrial plants required fundamental adaptations of developmental programs that were realized by cooperative actions of different plant hormones. Establishment of the corresponding signaling systems was indispensable for hormonal functioning. Domain-based phylogenetic studies of Gruhn and others (2014) and a search for orthologs of the CHASE domain-containing AHKs suggest that CK signaling originated in charophyte lineages, where also auxin and strigolactone signaling might have emerged (Wang and others 2015). This indicates that the development of signaling systems for these three phytohormones has been essential for development of multicellular plant organisms and probably was also associated with the early stages of their invasion of the land (Pils and Heyl 2009). On the other hand, signaling pathways of plant hormones involved in the control of plant responses to biotic and abiotic stresses (abscisic acid, jasmonic acid and salicylic acid) might have emerged in plants exposed to stress conditions while they colonized the terrestrial land (Wang and others 2015).

Back to the Beginning: Probing the Biological Functions of Cytokinins in Plants Overexpressing Genes Involved in Cytokinin Biosynthesis and Degradation

The early studies of the biological roles of CKs were mostly based on application of CKs to intact plants, detached plant parts, or plant tissue cultures. In spite of the fact that individual plant hormones, including CKs, act in cooperation/interaction with other phytohormones, this simple approach demonstrated the promoting effects of CKs on cell division, budding and lateral shoot formation, enhancement of metabolic sinks, as well as inhibition of apical dominance, root development and the progress of post-mitotic senescence in detached plant parts (see Kende 1971; Hall 1973; Mok 1994). Identification of genes encoding CK biosynthetic (IPT) and degradation (CKX) enzymes, as well as of components of CK signaling systems, has enabled construction of transgenic plants with altered levels of endogenous CKs and their molecular recognition. This has allowed testing of the functioning of CKs *in planta. T*he effects of changes in endogenous CK levels in genetically modified plants confirmed the results obtained by exogenous application of CKs to plants in the early days of CK research: Reduction of the CK levels in *Arabidopsis* plants following overexpression of different CKX genes resulted in reduced shoot development and enhanced root growth, consistent with the hypothesis that CKs have central, but opposite, regulatory functions in root and shoot meristems (Werner and others 2003). Expression of *IPT* under the control of a senescence-inducible promotor *SAG12* suppressed leaf senescence in *Arabidopsis* and several other plant species in a negatively autoregulatory manner (Gan and Amasino 1995; Jordi and others 2000; Sýkorová and others 2008). Correspondingly, leaves of CK deficient tobacco plants overexpressing CKX showed drastically reduced sink strength, as indicated by lower contents of soluble sugars and ATP as well as decreased activities of vacuolar invertases (Werner and others 2008). Disruption of CK signaling in *Arabidopsis* by creating double mutants in receptors *AHK*2 and *AHK3*

resulted in reduced leaf size and a semidwarf phenotype (Nishimura and others 2004).

Personal Recollections

Participating in plant hormone research for the last 50 years has given me the opportunity to meet and work with many interesting people who shared one common character—curiosity. Their often very different life stories include tales and events which will be lost if not shared. I was lucky to work for long periods of time with very interesting people involved in plant hormone research like Profs Donald Armstrong (Oregon State University), Robert Bock (University of Wisconsin), Roy Morris (University of Missouri) and Eugene Fox (ARCO Plant Cell Research Institute, California). With respect to limited space provided me here I can mention only a few memories of Prof Folke Skoog who was an inspiring person for many of us not only in plant science.

Identification of kinetin was only one of several remarkable discoveries in the field of plant biology achieved by Prof Folke Skoog and his associates (see Armstrong 2002, for his memories and a tribute to F.S.). I met Prof Folke Skoog in June 1968 when he visited Prague just before the Russian invasion, and I then spent almost 2 years in his laboratory as a research associate. Prof Skoog had an unusual instinct for identifying principal problems in plant developmental biology and the ability to link this to astute choices of appropriate approaches to their solution. However, he also experienced some life-changing events in his time. Representing Sweden in the 1932 Olympic Games in the 1500 m run, he did not win though he had the best time in trials. As a visitor to the next Olympics in 1936 in Berlin, he was seated as a guest on the main platform just a few rows behind Adolf Hitler. There, according to his own words, he missed a once in a lifetime opportunity: "I was a good shot and could not have missed at such a short range!"

During my stay in Madison in 1970, on August 24 shortly after midnight, young opponents of the war in Vietnam blew up a truck filled with explosives in the narrow street between the Mathematics and Botany departments, supposing that some military research was being performed in the former. The Botany laboratory and the greenhouse on the other site of the building were heavily damaged, including a crop of the well-known tobacco plants cv. Wisconsin 38 (Fig. 4). Early the next morning, we rummaged a ringing phone out from plaster fragments in the laboratory. Prof Skoog was calling from a conference asking what had happed to his laboratory. "Dr Skoog, you have no lab any more" answered my colleague John Burrows. It was a custom that students and post-doctorate fellows would hang their coffee cups marked

Fig. 4 Devastation of the Departments of Botany (*right*) and Mathematics (*left*), University of Wisconsin, Madison, USA, August 24th 1970

with their names on a pipe under the ceiling when their stay in the laboratory terminated. Almost all the cups were destroyed by the explosion. Looking on the few remaining cups Prof Skoog said: "So only John, Don and… survived. I have always been convinced that these boys there are sturdy". Prof Skoog was known for his special kind of dry humor…

After the Botanical Congress that was held in Seattle in 1970, Prof Chailachian from Moscow, who introduced the concept of florigen to plant biology, visited the laboratory guided by Prof Skoog and an interpreter. When they came to my bench I spoke to him in Russian. Leaving the laboratory, the guest said that I was the first American he had met speaking Russian so well. The next day Prof Skoog commented: "There was no need to perturb his good opinion about us!"

Prof Skoog several times attended symposia and conferences devoted to plant hormones organized by the Institute of Experimental Botany in the Czech Republic and was often our guest in Prague. Once he suffered from a stomach problem. A tea made from lady's mantle (*Alchemilla mollis*), prepared for him by my wife Jana, finally helped. In gratitude, he later handed her a present brought for her from Madison, a bottle of the perfume White Witch. He brought a certain magic to all who worked with him as well.

General Remarks

Similarly to other fields of plant science, the research of CKs is more and more performed on a cooperative basis that is dependent on exploitation of advanced sophisticated methods and the availability of correspondingly expensive equipment. This cogent trend generates enormous amounts of valuable data often at the expense of the satisfaction of

our curiosity and joy in science as an intellectual activity. Taking this into account, there is no need to be sorry for those researchers who laid down the basis of research on CKs and plant hormones in general under very simple and often difficult conditions…

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