Chapter

21

Cytokinins:
Regulators of Cell Division

THE CYTOKININS WERE DISCOVERED in the search for factors that stimulate plant cells to divide (i.e., undergo cytokinesis). Since their discovery, cytokinins have been shown to have effects on many other physiological and developmental processes, including leaf senescence, nutrient mobilization, apical dominance, the formation and activity of shoot apical meristems, floral development, the breaking of bud dormancy, and seed germination. Cytokinins also appear to mediate many aspects of light-regulated development, including chloroplast differentiation, the development of autotrophic metabolism, and leaf and cotyledon expansion.

Although cytokinins regulate many cellular processes, the control of cell division is central in plant growth and development and is considered diagnostic for this class of plant growth regulators. For these reasons we will preface our discussion of cytokinin function with a brief consideration of the roles of cell division in normal development, wounding, gall formation, and tissue culture.

Later in the chapter we will examine the regulation of plant cell proliferation by cytokinins. Then we will turn to cytokinin functions not directly related to cell division: chloroplast differentiation, the prevention of leaf senescence, and nutrient mobilization. Finally, we will consider the molecular mechanisms underlying cytokinin perception and signaling.

CELL DIVISION AND PLANT DEVELOPMENT

Plant cells form as the result of cell divisions in a primary or secondary meristem. Newly formed plant cells typically enlarge and differentiate, but once they have assumed their function—whether transport, photosynthesis, support, storage, or protection—usually they do not divide again during the life of the plant. In this respect they appear to be similar to animal cells, which are considered to be terminally differentiated.

However, this similarity to the behavior of animal cells is only superficial. Almost every type of plant cell that retains its nucleus at maturity

has been shown to be capable of dividing. This property comes into play during such processes as wound healing and leaf abscission.

Differentiated Plant Cells Can Resume Division

Under some circumstances, mature, differentiated plant cells may resume cell division in the intact plant. In many species, mature cells of the cortex and/or phloem resume division to form secondary meristems, such as the vascular cambium or the cork cambium. The abscission zone at the base of a leaf petiole is a region where mature parenchyma cells begin to divide again after a period of mitotic inactivity, forming a layer of cells with relatively weak cell walls where abscission can occur (see Chapter 22).

Wounding of plant tissues induces cell divisions at the wound site. Even highly specialized cells, such as phloem fibers and guard cells, may be stimulated by wounding to divide at least once. Wound-induced mitotic activity typically is self-limiting; after a few divisions the derivative cells stop dividing and redifferentiate. However, when the soil-dwelling bacterium *Agrobacterium tumefaciens* invades a wound, it can cause the neoplastic (tumor-forming) disease known as crown gall. This phenomenon is dramatic natural evidence of the mitotic potential of mature plant cells.

Without *Agrobacterium* infection, the wound-induced cell division would subside after a few days and some of the new cells would differentiate as a protective layer of cork cells or vascular tissue. However, *Agrobacterium* changes the character of the cells that divide in response to the wound, making them tumorlike. They do not stop dividing; rather they continue to divide throughout the life of the plant to produce an unorganized mass of tumorlike tissue called a **gall** (Figure 21.1). We will have more to say about this important disease later in this chapter.
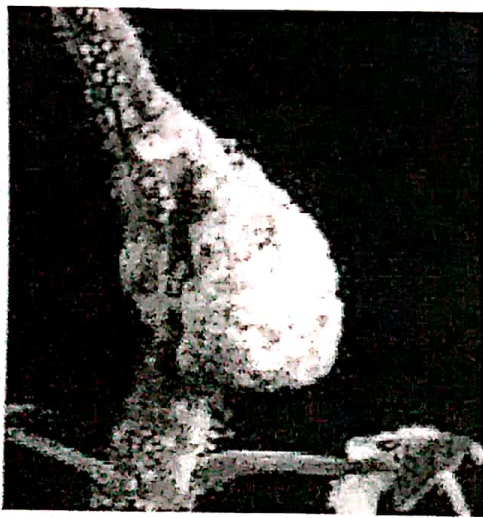

FIGURE 21.1   Tumor that formed on a tomato stem infected with the crown gall bacterium, *Agrobacterium tumefaciens*. Two months before this photo was taken the stem was wounded and inoculated with a virulent strain of the crown gall bacterium. (From Aloni et al. 1998, courtesy of R. Aloni.)

Diffusible Factors May Control Cell Division

The considerations addressed in the previous section suggest that mature plant cells stop dividing because they no longer receive a particular signal, possibly a hormone, that is necessary for the initiation of cell division. The idea that cell division may be initiated by a diffusible factor originated with the Austrian plant physiologist G. Haberlandt, who, in about 1913, demonstrated that vascular tissue contains a water-soluble substance or substances that will stimulate the division of wounded potato tuber tissue. The effort to determine the nature of this factor (or factors) led to the discovery of the cytokinins in the 1950s.

Plant Tissues and Organs Can Be Cultured

Biologists have long been intrigued by the possibility of growing organs, tissues, and cells in culture on a simple nutrient medium, in the same way that microorganisms can be cultured in test tubes or on petri dishes. In the 1930s, Philip White demonstrated that tomato roots can be grown indefinitely in a simple nutrient medium containing only sucrose, mineral salts, and a few vitamins, with no added hormones (White 1934).

In contrast to roots, isolated stem tissues exhibit very little growth in culture without added hormones in the medium. Even if auxin is added, only limited growth may occur, and usually this growth is not sustained. Frequently this auxin-induced growth is due to cell enlargement only. The shoots of most plants cannot grow on a simple medium lacking hormones, even if the cultured stem tissue contains apical or lateral meristems, until adventitious roots form. Once the stem tissue has rooted, shoot growth resumes, but now as an integrated, whole plant.

These observations indicate that there is a difference in the regulation of cell division in root and shoot meristems. They also suggest that some root-derived factor(s) may regulate growth in the shoot.

Crown gall stem tissue is an exception to these generalizations. After a gall has formed on a plant, heating the plant to 42°C will kill the bacterium that induced gall formation. The plant will survive the heat treatment, and its gall tissue will continue to grow as a bacteria-free tumor (Braun 1958).

Tissues removed from these bacteria-free tumors grow on simple, chemically defined culture media that would not support the proliferation of normal stem tissue of the same species. However, these stem-derived tissues are not organized. Instead they grow as a mass of disorganized, relatively undifferentiated cells called callus tissue.

Callus tissue sometimes forms naturally in response to wounding, or in graft unions where stems of two different plants are joined. Crown gall tumors are a specific type of callus, whether they are growing attached to the plant or in culture. The finding that crown gall callus tissue can be cultured demonstrated that cells derived from stem tissues are capable of proliferating in culture and that contact with

the bacteria may cause the stem cells to produce cell division–stimulating factors.

THE DISCOVERY, IDENTIFICATION, AND PROPERTIES OF CYTOKININS

A great many substances were tested in an effort to initiate and sustain the proliferation of normal stem tissues in culture. Materials ranging from yeast extract to tomato juice were found to have a positive effect, at least with some tissues. However, culture growth was stimulated most dramatically when the liquid endosperm of coconut, also known as coconut milk, was added to the culture medium.

Philip White's nutrient medium, supplemented with an auxin and 10 to 20% coconut milk, will support the continued cell division of mature, differentiated cells from a wide variety of tissues and species, leading to the formation of callus tissue (Caplin and Steward 1948). This finding indicated that coconut milk contains a substance or substances that stimulate mature cells to enter and remain in the cell division cycle.

Eventually coconut milk was shown to contain the cytokinin *zeatin*, but this finding was not obtained until several years after the discovery of the cytokinins (Letham 1974). The first cytokinin to be discovered was the synthetic analog kinetin.

Kinetin Was Discovered as a Breakdown Product of DNA

In the 1940s and 1950s, Folke Skoog and coworkers at the University of Wisconsin tested many substances for their ability to initiate and sustain the proliferation of cultured tobacco pith tissue. They had observed that the nucleic acid base adenine had a slight promotive effect, so they tested the possibility that nucleic acids would stimulate division in this tissue. Surprisingly, autoclaved herring sperm DNA had a powerful cell division–promoting effect.

After much work, a small molecule was identified from the autoclaved DNA and named **kinetin**. It was shown to be an adenine (or aminopurine) derivative, 6-furfurylaminopurine (Miller et al. 1955):
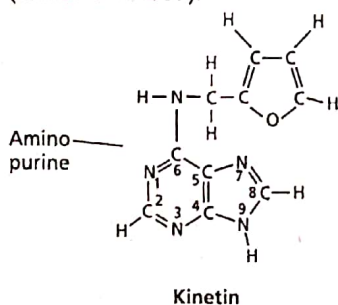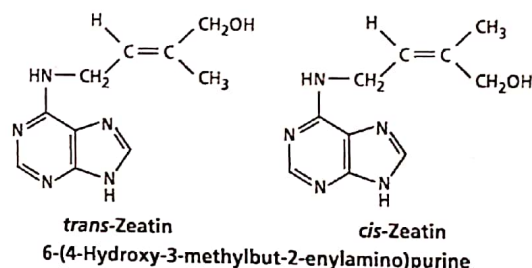

Kinetin

In the presence of an auxin, kinetin would stimulate tobacco pith parenchyma tissue to proliferate in culture. No kinetin-induced cell division occurs without auxin in the culture medium. (For more details, see Web Topic 21.1.)

Kinetin is not a naturally occurring plant growth regulator, and it does not occur as a base in the DNA of any species. It is a by-product of the heat-induced degradation of DNA, in which the deoxyribose sugar of adenosine is converted to a furfuryl ring and shifted from the 9 position to the 6 position on the adenine ring.

The discovery of kinetin was important because it demonstrated that cell division could be induced by a simple chemical substance. Of greater importance, the discovery of kinetin suggested that naturally occurring molecules with structures similar to that of kinetin regulate cell division activity within the plant. This hypothesis proved to be correct.

Zeatin Is the Most Abundant Natural Cytokinin

Several years after the discovery of kinetin, extracts of the immature endosperm of corn (*Zea mays*) were found to contain a substance that has the same biological effect as kinetin. This substance stimulates mature plant cells to divide when added to a culture medium along with an auxin. Letham (1973) isolated the molecule responsible for this activity and identified it as *trans*-6-(4-hydroxy-3-methylbut-2-enylamino)purine, which he called **zeatin**:

trans-Zeatin   **cis-Zeatin**
6-(4-Hydroxy-3-methylbut-2-enylamino)purine

The molecular structure of zeatin is similar to that of kinetin. Both molecules are adenine or aminopurine derivatives. Although they have different side chains, in both cases the side chain is attached to the 6 nitrogen of the aminopurine. Because the side chain of zeatin has a double bond, it can exist in either the *cis* or the *trans* configuration.

In higher plants, zeatin occurs in both the *cis* and the *trans* configurations, and these forms can be interconverted by an enzyme known as *zeatin isomerase*. Although the *trans* form of zeatin is much more active in biological assays, the *cis* form may also play important roles, as suggested by the fact that it has been found in high levels in a number of plant species and particular tissues. A gene encoding a glucosyl transferase enzyme specific to *cis*-zeatin has recently been cloned, which further supports a biological role for this isoform of zeatin.

Since its discovery in immature maize endosperm, zeatin has been found in many plants and in some bacteria. It is the most prevalent cytokinin in higher plants, but other substituted aminopurines that are active as cytokinins have been isolated from many plant and bac-

terial species. These aminopurines differ from zeatin in the nature of the side chain attached to the 6 nitrogen or in the attachment of a side chain to carbon 2:

N⁶-(Δ²-Isopentenyl)-adenine (iP)

Dihydrozeatin (DZ)
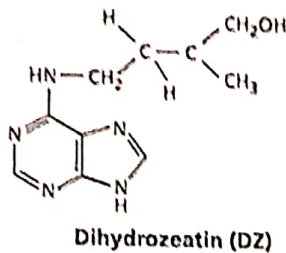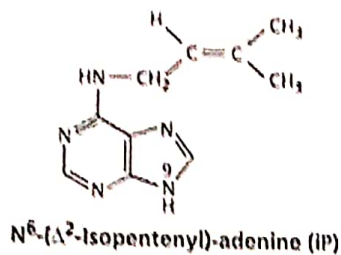
In addition, these cytokinins can be present in the plant as a **riboside** (in which a ribose sugar is attached to the 9 nitrogen of the purine ring), a **ribotide** (in which the ribose sugar moiety contains a phosphate group), or a **glycoside** (in which a sugar molecule is attached to the 3, 7, or 9 nitrogen of the purine ring, or to the oxygen of the zeatin or dihydrozeatin side chain) (see Web Topic 21.2).

## Some Synthetic Compounds Can Mimic or Antagonize Cytokinin Action

Cytokinins are defined as compounds that have biological activities similar to those of *trans*-zeatin. These activities include the ability to do the following:

- Induce cell division in callus cells in the presence of an auxin
- Promote bud or root formation from callus cultures when in the appropriate molar ratios to auxin
- Delay senescence of leaves
- Promote expansion of dicot cotyledons

Many chemical compounds have been synthesized and tested for cytokinin activity. Analysis of these compounds provides insight into the structural requirements for activity. Nearly all compounds active as cytokinins are N⁶-substituted aminopurines, such as benzyladenine (BA):

Benzyladenine
(benzylaminopurine)
(BA)

and all the naturally occurring cytokinins are aminopurine derivatives. There are also synthetic cytokinin compounds that have not been identified in plants, most notable of which are the diphenylurea-type cytokinins, such as thidiazuron, which is used commercially as a defoliant and an herbicide:

N,N'-Diphenylurea (nonamino purine with weak activity)

Thidiazuron
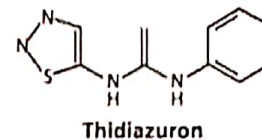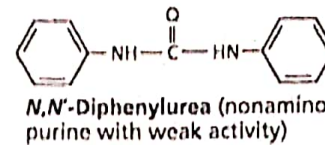
In the course of determining the structural requirements for cytokinin activity, investigators found that some molecules act as *cytokinin antagonists*:

3-Methyl-7-(3-methylbutylamino)pyrazolo[4,3-D]pyrimidine
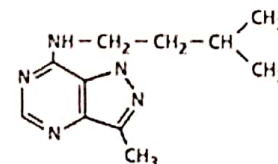
These molecules are able to block the action of cytokinins, and their effects may be overcome by the addition of more cytokinin. Naturally occurring molecules with cytokinin activity may be detected and identified by a combination of physical methods and bioassays (see Web Topic 21.3).

Cytokinins Occur in Both Free and Bound Forms

Hormonal cytokinins are present as free molecules (not covalently attached to any macromolecule) in plants and certain bacteria. Free cytokinins have been found in a wide spectrum of angiosperms and probably are universal in this group of plants. They have also been found in algae, diatoms, mosses, ferns, and conifers.

The regulatory role of cytokinins has been demonstrated only in angiosperms, conifers, and mosses, but they may function to regulate the growth, development, and metabolism of all plants. Usually zeatin is the most abundant naturally occurring free cytokinin, but *dihydrozeatin* (DZ) and *isopentenyl adenine* (iP) also are commonly found in higher plants and bacteria. Numerous derivatives of these three cytokinins have been identified in plant extracts (see the structures illustrated in Figure 21.6).

Transfer RNA (tRNA) contains not only the four nucleotides used to construct all other forms of RNA, but also some unusual nucleotides in which the base has been modified. Some of these "hypermodified" bases act as cytokinins when the tRNA is hydrolyzed and tested in one of the cytokinin bioassays. Some plant tRNAs contain *cis-*

zeatin as a hypermodified base. However, cytokinins are not confined to plant tRNAs. They are part of certain tRNAs from all organisms, from bacteria to humans. (For details, see Web Topic 21.4.)

The Hormonally Active Cytokinin Is the Free Base

It has been difficult to determine which species of cytokinin represents the active form of the hormone, but the recent identification of the cytokinin receptor CRE1 has allowed this question to be addressed. The relevant experiments have shown that the free-base form of *trans*-zeatin, but not its ribo-side or ribotide derivatives, binds directly to CRE1, indicating that the free base is the active form (Yamada et al. 2001).

Although the free-base form of *trans*-zeatin is thought to be the hormonally active cytokinin, some other compounds have cytokinin activity, either because they are readily converted to zeatin, dihydrozeatin, or isopentenyl adenine, or because they release these compounds from other molecules, such as cytokinin glucosides. For example, tobacco cells in culture do not grow unless cytokinin ribosides supplied in the culture medium are converted to the free base.

In another example, excised radish cotyledons grow when they are cultured in a solution containing the cytokinin base benzyladenine (BA, an N^6-substituted aminopurine cytokinin). The cultured cotyledons readily take up the hormone and convert it to various BA glucosides, BA ribonucleoside, and BA ribonucleotide. When the cotyledons are transferred back to a medium lacking a cytokinin, their growth rate declines, as do the concentrations of BA, BA ribonucleoside, and BA ribonucleotide in the tissues. However, the level of the BA glucosides remains constant. This finding suggests that the glucosides cannot be the active form of the hormone.

Some Plant Pathogenic Bacteria, Insects, and Nematodes Secrete Free Cytokinins

Some bacteria and fungi are intimately associated with higher plants. Many of these microorganisms produce and secrete substantial amounts of cytokinins and/or cause the plant cells to synthesize plant hormones, including cytokinins (Akiyoshi et al. 1987). The cytokinins produced by microorganisms include *trans*-zeatin, [9R]iP, *cis*-zeatin, and their ribosides (Figure 21.2). Infection of plant tissues with these microorganisms can induce the tissues to divide and, in some cases, to form special structures, such as mycorrhizae, in which the microorganism can reside in a mutualistic relationship with the plant.

In addition to the crown gall bacterium, *Agrobacterium tumefaciens*, other pathogenic bacteria may stimulate plant cells to divide. For example, *Corynebacterium fascians* is a major cause of the growth abnormality known as **witches'-broom** (Figure 21.3). The shoots of plants infected by *C. fascians* resemble an old-fashioned straw broom because the lateral buds, which normally remain dormant, are stimulated by the bacterial cytokinin to grow (Hamilton and Lowe 1972).
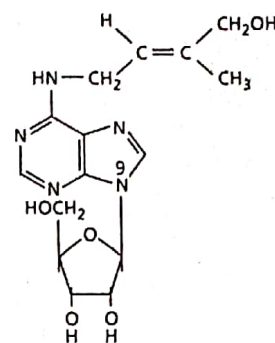
Ribosylzeatin (zeatin riboside)
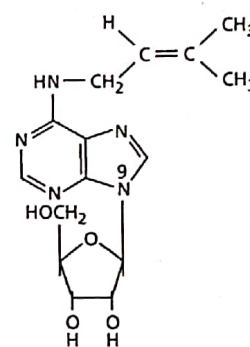
N^6-(Δ^2-Isopentenyl)adenosine ([9R]iP)

FIGURE 21.2   Structures of ribosylzeatin and N6-(Δ2-isopentenyl)adenosine ([9R]iP).

FIGURE 21.3   Witches' broom on balsam fir (*Abies balsamea*). (Photo © Gregory K. Scott/Photo Researchers, Inc.)

Infection with a close relative of the crown gall organism, *Agrobacterium rhizogenes*, causes masses of roots instead of callus tissue to develop from the site of infection. *A. rhizogenes* is able to modify cytokinin metabolism in infected plant tissues through a mechanism that will be described later in this chapter.

Certain insects secrete cytokinins, which may play a role in the formation of galls utilized by these insects as feeding sites. Root-knot nematodes also produce cytokinins, which may be involved in manipulating host development to produce the giant cells from which the nematode feeds (Elzen 1983).

BIOSYNTHESIS, METABOLISM, AND TRANSPORT OF CYTOKININS

The side chains of naturally occurring cytokinins are chemically related to rubber, carotenoid pigments, the plant hormones gibberellin and abscisic acid, and some of the plant defense compounds known as phytoalexins. All of these compounds are constructed, at least in part, from isoprene units (see Chapter 13).

Isoprene is similar in structure to the side chains of zeatin and iP (see the structures illustrated in Figure 21.6). These cytokinin side chains are synthesized from an isoprene derivative. Large molecules of rubber and the carotenoids are constructed by the polymerization of many isoprene units; cytokinins contain just one of these units. The precursor(s) for the formation of these isoprene structures are either mevalonic acid or pyruvate plus 3-phosphoglycerate, depending on which pathway is involved (see Chapter 13). These precursors are converted to the biological isoprene unit dimethylallyl diphosphate (DMAPP).

Crown Gall Cells Have Acquired a Gene for Cytokinin Synthesis

Bacteria-free tissues from crown gall tumors proliferate in culture without the addition of any hormones to the culture medium. Crown gall tissues contain substantial amounts of both auxin and free cytokinins. Furthermore, when radioactively labeled adenine is fed to periwinkle (*Vinca rosea*) crown gall tissues, it is incorporated into both zeatin and zeatin riboside, demonstrating that gall tissues contain the cytokinin biosynthetic pathway. Control stem tissue, which has not been transformed by *Agrobacterium*, does not incorporate labeled adenine into cytokinins.

During infection by *Agrobacterium tumefaciens*, plant cells incorporate bacterial DNA into their chromosomes. The virulent strains of *Agrobacterium* contain a large plasmid known as the **Ti plasmid**. Plasmids are circular pieces of extrachromosomal DNA that are not essential for the life of the bacterium. However, plasmids frequently contain genes that enhance the ability of the bacterium to survive in special environments.

A small portion of the Ti plasmid, known as the T-DNA, is incorporated into the nuclear DNA of the host plant cell (Figure 21.4) (Chilton et al. 1977). T-DNA carries genes necessary for the biosynthesis of *trans*-zeatin and auxin, as well as a member of a class of unusual nitrogen-containing compounds called *opines* (Figure 21.5). Opines are not synthesized by plants except after crown gall transformation.

The T-DNA gene involved in cytokinin biosynthesis—known as the *ipt*[1] gene—encodes an *isopentenyl transferase* (IPT) enzyme that transfers the isopentenyl group from DMAPP to AMP (adenosine monophosphate) to form isopentenyl adenine ribotide (Figure 21.6) (Akiyoshi et al. 1984; Barry et al. 1984). The *ipt* gene has been called the *tmr* locus because, when *inactivated* by mutation, it results in "rooty" tumors. Isopentenyl adenine ribotide can be converted to the active cytokinins isopentenyl adenine, *trans*-zeatin, and dihydrozeatin by endogenous enzymes in plant cells. This conversion route is similar to the pathway for cytokinin synthesis that has been postulated for normal tissue (see Figure 21.6).

The T-DNA also contains two genes encoding enzymes that convert tryptophan to the auxin indole-3-acetic acid (IAA). This pathway of auxin biosynthesis differs from the one in nontransformed cells and involves indoleacetamide as an intermediate (see Figure 19.6). The *ipt* gene and the two auxin biosynthetic genes of T-DNA are **phyto-oncogenes**, since they can induce tumors in plants (see Web Topic 21.5).

Because their promoters are plant eukaryotic promoters, none of the T-DNA genes are expressed in the bacterium; rather they are transcribed after they are inserted into the plant chromosomes. Transcription of the genes leads to synthesis of the enzymes they encode, resulting in the production of zeatin, auxin, and an opine. The bacterium can utilize the opine as a nitrogen source, but cells of higher plants cannot. Thus, by transforming the plant cells, the bacterium provides itself with an expanding environment (the gall tissue) in which the host cells are directed to produce a substance (the opine) that only the bacterium can utilize for its nutrition (Bomhoff et al. 1976).

An important difference between the control of cytokinin biosynthesis in crown gall tissues and in normal tissues is that the T-DNA genes for cytokinin synthesis are expressed in all infected cells, even those in which the native plant genes for biosynthesis of the hormone are normally repressed.

IPT Catalyzes the First Step in Cytokinin Biosynthesis

The first committed step in cytokinin biosynthesis is the transfer of the isopentenyl group of dimethylallyl diphos-

---

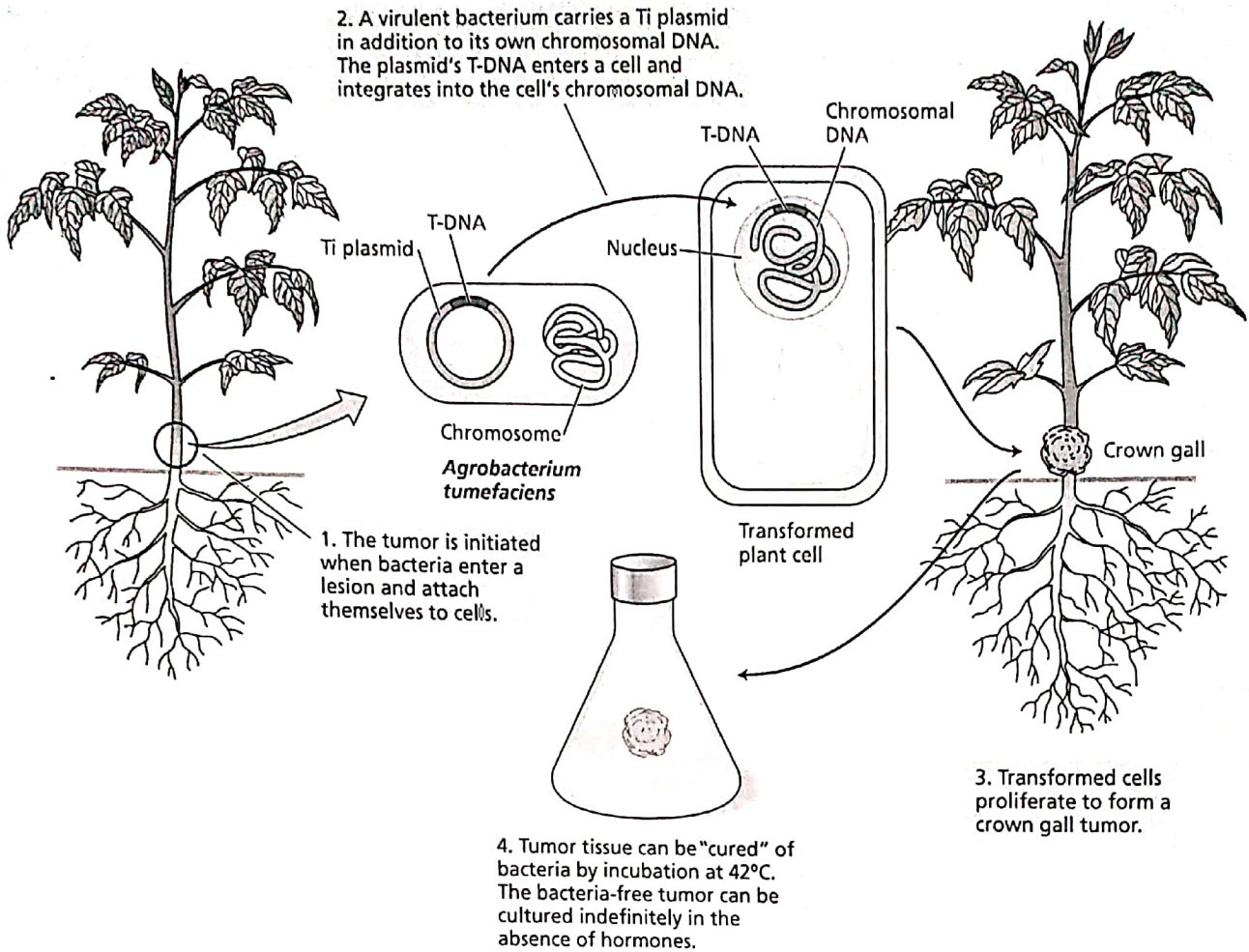[1] Bacterial genes, unlike plant genes, are written in lowercase italics.

FIGURE 21.4   Tumor induction by *Agrobacterium tumefaciens*. (After Chilton 1983.)

phate (DMAPP) to an adenosine moiety. An enzyme that catalyzes such an activity was first identified in the cellular slime mold *Dictyostelium discoideum*, and subsequently the *ipt* gene from *Agrobacterium* was found to encode such an enzyme. In both cases, DMAPP and AMP are converted to isopentenyladenosine-5'-monophosphate (iPMP).

As noted earlier, cytokinins are also present in the tRNAs of most cells, including plant and animal cells. The tRNA cytokinins are synthesized by modification of specific adenine residues within the fully transcribed tRNA. As with the free cytokinins, isopentenyl groups are transferred to the adenine molecules from DMAPP by an enzyme call tRNA-IPT. The genes for tRNA-IPT have been cloned from many species.
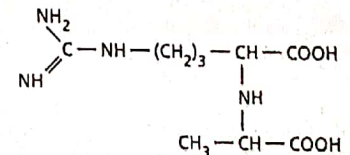
Octopine
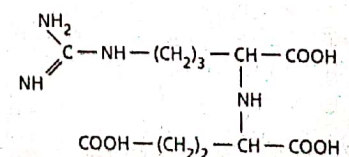
Nopaline

FIGURE 21.5   The two major opines, octopine and nopaline, are found only in crown gall tumors. The genes required for their synthesis are present in the T-DNA from *Agrobacterium tumefaciens*. The bacterium, but not the plant, can utilize the opines as a nitrogen source.
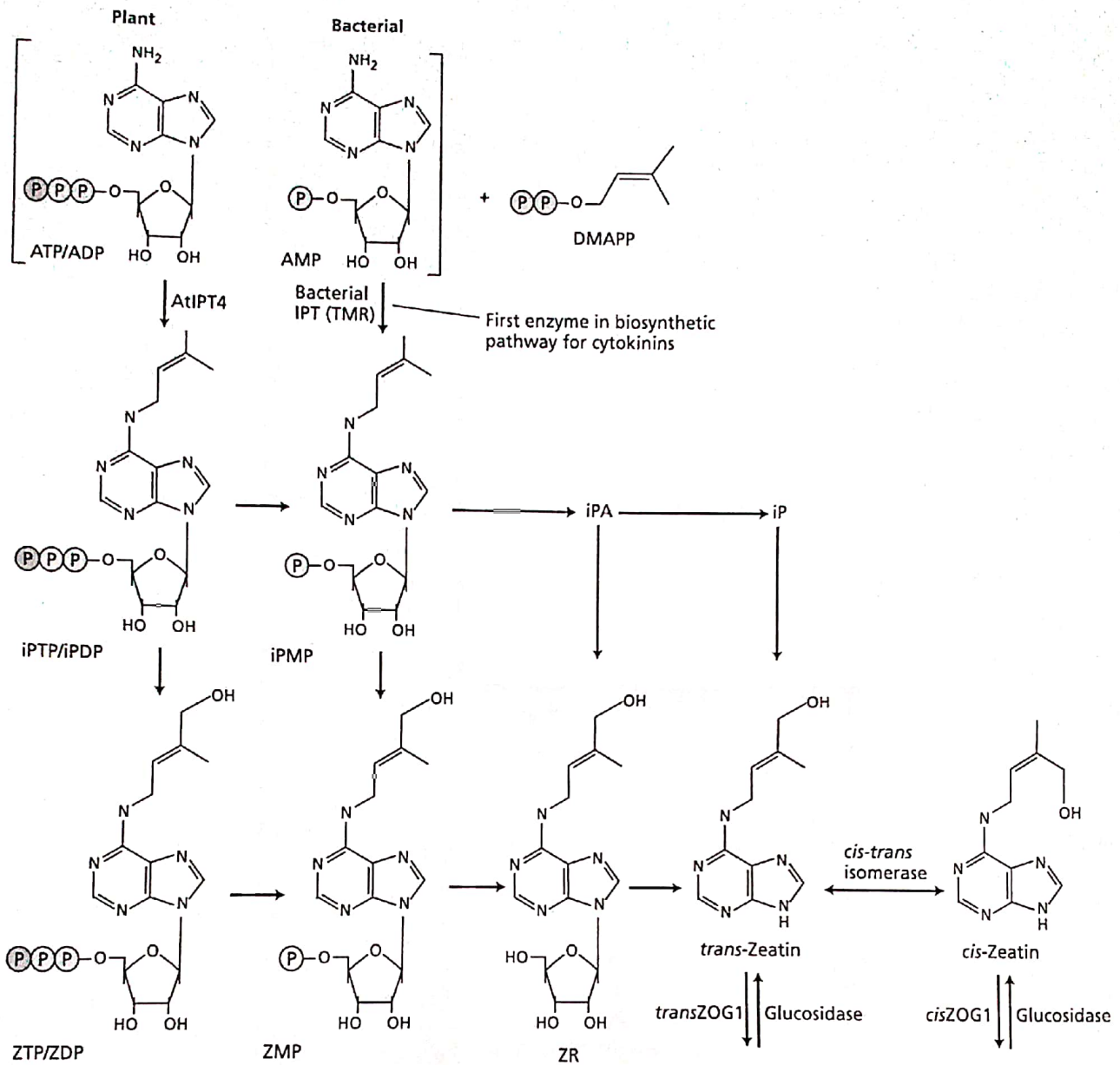
FIGURE 21.6 Biosynthetic pathway for cytokinin biosynthesis. The first committed step in cytokinin biosynthesis is the addition of the isopentenyl side chain from DMAPP to an adenosine moiety. The plant and bacterial IPT enzymes differ in the adenosine substrate used; the plant enzyme appears to utilize both ADP and ATP, and the bacterial enzyme utilizes AMP. The products of these reactions (iPMP, iPDP, or iPTP) are converted to zeatin by an unidentified hydroxylase. The various phosphorylated forms can be interconverted and free *trans*-Zeatin can be formed from the riboside by enzymes of general purine metabolism. *trans*-Zeatin can be metabolized in various ways as shown, and these reactions are catalyzed by the indicated enzymes.
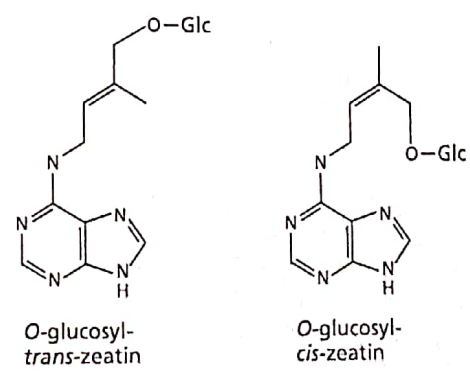
The possibility that free cytokinins are derived from tRNA has been explored extensively. Although the tRNA-bound cytokinins can act as hormonal signals for plant cells if the tRNA is degraded and fed back to the cells, it is unlikely that any significant amount of the free hormonal cytokinin in plants is derived from the turnover of tRNA.

An enzyme with IPT activity was identified from crude extracts of various plant tissues, but researchers were unable to purify the protein to homogeneity. Recently, plant IPT genes were cloned after the *Arabidopsis* genome was analyzed for potential *ipt*-like sequences (Kakimoto 2001; Takei et al. 2001). Nine different *IPT* genes were identified

in *Arabidopsis*—many more than are present in animal genomes, which generally contain only one or two such genes used in tRNA modification.

Phylogenetic analysis revealed that one of the *Arabidopsis IPT* genes resembles bacterial tRNA-*ipt*, another resembles eukaryotic tRNA-*IPT*, and the other seven form a distinct group or clade together with other plant sequences (see Web Topic 21.6). The grouping of the seven *Arabidopsis IPT* genes in this unique plant clade provided a clue that these genes may encode the cytokinin biosynthetic enzyme.

The proteins encoded by these genes were expressed in *E. coli* and analyzed. It was found that, with the exception of the gene most closely related to the animal tRNA-*IPT* genes, these genes encoded proteins capable of synthesizing free cytokinins. Unlike their bacterial counterparts, however, the *Arabidopsis* enzymes that have been analyzed utilize ATP and ADP preferentially over AMP (see Figure 21.6).

Cytokinins from the Root Are Transported to the Shoot via the Xylem

Root apical meristems are major sites of synthesis of the free cytokinins in whole plants. The cytokinins synthesized in roots appear to move through the xylem into the shoot, along with the water and minerals taken up by the roots. This pathway of cytokinin movement has been inferred from the analysis of xylem exudate.

When the shoot is cut from a rooted plant near the soil line, the xylem sap may continue to flow from the cut stump for some time. This xylem exudate contains cytokinins. If the soil covering the roots is kept moist, the flow of xylem exudate can continue for several days. Because the cytokinin content of the exudate does not diminish, the cytokinins found in it are likely to be synthesized by the roots. In addition, environmental factors that interfere with root function, such as water stress, reduce the cytokinin content of the xylem exudate (Itai and Vaadia 1971). Conversely, resupply of nitrate to nitrogen-starved maize roots results in an elevation of the concentration of cytokinins in the xylem sap (Samuelson 1992), which has been correlated to an induction of cytokinin-regulated gene expression in the shoots (Takei et al. 2001).

Although the presence of cytokinin in the xylem is well established, recent grafting experiments have cast doubt on the presumed role of this root-derived cytokinin in shoot development. Tobacco transformed with an inducible *ipt* gene from *Agrobacterium* displayed increased lateral bud outgrowth and delayed senescence.

To assess the role of cytokinin derived from the root, the tobacco root stock engineered to overproduce cytokinin was grafted to a wild-type shoot. Surprisingly, no phenotypic consequences were observed in the shoot, even though an increased concentration of cytokinin was measured in the transpiration stream (Faiss et al. 1997). Thus the excess cytokinin in the roots had no effect on the grafted shoot.

Roots are not the only parts of the plant capable of synthesizing cytokinins. For example, young maize embryos synthesize cytokinins, as do young developing leaves, young fruits, and possibly many other tissues. Clearly, further studies will be needed to resolve the roles of cytokinins transported from the root versus cytokinins synthesized in the shoot.

A Signal from the Shoot Regulates the Transport of Zeatin Ribosides from the Root

The cytokinins in the xylem exudate are mainly in the form of zeatin ribosides. Once they reach the leaves, some of these nucleosides are converted to the free-base form or to glucosides (Noodén and Letham 1993). Cytokinin glucosides may accumulate to high levels in seeds and in leaves, and substantial amounts may be present even in senescing leaves. Although the glucosides are active as cytokinins in bioassays, often they lack hormonal activity after they form within cells, possibly because they are compartmentalized in such a way that they are unavailable. Compartmentation may explain the conflicting observations that cytokinins are transported readily by the xylem but that radioactive cytokinins applied to leaves in intact plants do not appear to move from the site of application.

Evidence from grafting experiments with mutants suggests that the transport of zeatin riboside from the root to the shoot is regulated by signals from the shoot. The *rms4* mutant of pea (*Pisum sativum* L.) is characterized by a 40-fold decrease in the concentration of zeatin riboside in the xylem sap of the roots. However, grafting a wild-type shoot onto an *rms4* mutant root increased the zeatin riboside levels in the xylem exudate to wild-type levels. Conversely, grafting an *rms4* mutant shoot onto a wild-type root lowered the concentration of zeatin riboside in the xylem exudate to mutant levels (Beveridge et al. 1997).

These results suggest that a signal from the shoot can regulate cytokinin transport from the root. The identity of this signal has not yet been determined.

Cytokinins Are Rapidly Metabolized by Plant Tissues

Free cytokinins are readily converted to their respective nucleoside and nucleotide forms. Such interconversions likely involve enzymes common to purine metabolism.

Many plant tissues contain the enzyme **cytokinin oxidase**, which cleaves the side chain from zeatin (both *cis* and *trans*), zeatin riboside, iP, and their *N*-glucosides, but not their *O*-glucoside derivatives (Figure 21.7). However, dihydrozeatin and its conjugates are resistant to cleavage. Cytokinin oxidase irreversibly inactivates cytokinins, and it could be important in regulating or limiting cytokinin effects. The activity of the enzyme is induced by high cytokinin concentrations, due at least in part to an elevation of the RNA levels for a subset of the genes.
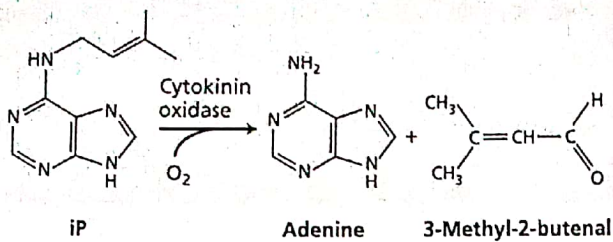
iP   Adenine   3-Methyl-2-butenal

FIGURE 21.7 Cytokinin oxidase irreversibly degrades some cytokinins.

A gene encoding cytokinin oxidase was first identified in maize (Houba-Herin et al. 1999; Morris et al. 1999). In *Arabidopsis*, cytokinin oxidase is encoded by a multigene family whose members show distinct patterns of expression. Interestingly, several of the genes contain putative secretory signals, suggesting that at least some of these enzymes may be extracellular.

Cytokinin levels can also be regulated by conjugation of the hormone at various positions. The nitrogens at the 3, 7, and 9 positions of the adenine ring of cytokinins can be conjugated to glucose residues. Alanine can also be conjugated to the nitrogen at the 9 positon, forming lupinic acid. These modifications are generally irreversible, and such conjugated forms of cytokinin are inactive in bioassays, with the exception of the N^3-glucosides.

The hydroxyl group of the side chain of cytokinins is also the target for conjugation to glucose residues, or in some cases xylose residues, yielding O-glucoside and O-xyloside cytokinins. O-glucosides are resistant to cleavage by cytokinin oxidases, which may explain why these derivatives have higher biological activity in some assays than their corresponding free bases have.

Enzymes that catalyze the conjugation of either glucose or xylose to zeatin have been purified, and their respective genes have been cloned (Martin et al. 1999). These enzymes have stringent substrate specificities for the sugar donor and the cytokinin bases. Only free *trans*-zeatin and dihydrozeatin bases are efficient substrates; the corresponding nucleosides are not substrates, nor is *cis*-zeatin. The specificity of these enzymes suggests that the conjugation to the side chain is precisely regulated.

The conjugations at the side chain can be removed by glucosidase enzymes to yield free cytokinins, which, as discussed earlier, are the active forms. Thus, cytokinin glucosides may be a storage form, or metabolically inactive state, of these compounds. A gene encoding a glucosidase that can release cytokinins from sugar conjugates has been cloned from maize, and its expression could play an important role in the germination of maize seeds (Brzobohaty et al. 1993).

Dormant seeds often have high levels of cytokinin glucosides but very low levels of hormonally active free cytokinins. Levels of free cytokinins increase rapidly, however, as germination is initiated, and this increase in free cytokinins is accompanied by a corresponding decrease in cytokinin glucosides.

THE BIOLOGICAL ROLES OF CYTOKININS

Although discovered as a cell division factor, cytokinins can stimulate or inhibit a variety of physiological, metabolic, biochemical, and developmental processes when they are applied to higher plants, and it is increasingly clear that endogenous cytokinins play an important role in the regulation of these events in the intact plant.

In this section we will survey some of the diverse effects of cytokinin on plant growth and development, including a discussion of its role in regulating cell division. The discovery of the tumor-inducing Ti plasmid in the plant-pathogenic bacterium *Agrobacterium tumefaciens* provided plant scientists with a powerful new tool for introducing foreign genes into plants, and for studying the role of cytokinin in development. In addition to its role in cell proliferation, cytokinin affects many other processes, including differentiation, apical dominance, and senescence.

Cytokinins Regulate Cell Division in Shoots and Roots

As discussed earlier, cytokinins are generally required for cell division of plant cells in vitro. Several lines of evidence suggest that cytokinins also play key roles in the regulation of cell division in vivo.

Much of the cell division in an adult plant occurs in the meristems (see Chapter 16). Localized expression of the *ipt* gene of *Agrobacterium* in somatic sectors of tobacco leaves causes the formation of ectopic (abnormally located) meristems, indicating that elevated levels of cytokinin are sufficient to initiate cell divisions in these leaves (Estruch et al. 1991). Elevation of endogenous cytokinin levels in transgenic *Arabidopsis* results in overexpression of the KNOTTED homeobox transcription factor homologs *KNAT1* and *STM*—genes that are important in the regulation of meristem function (see Chapter 16) (Rupp et al. 1999). Interestingly, overexpression of *KNAT1* also appears to elevate cytokinin levels in transgenic tobacco, suggesting an interdependent relationship between *KNAT* and the level of cytokinins.

Overexpression of several of the *Arabidopsis* cytokinin oxidase genes in tobacco results in a reduction of endogenous cytokinin levels and a consequent strong retardation of shoot development due to a reduction in the rate of cell proliferation in the shoot apical meristem (Figures 21.8 and 21.9) (Werner et al. 2001). This finding strongly supports the notion that endogenous cytokinins regulate cell division in vivo.

Surprisingly, the same overexpression of cytokinin oxidase in tobacco led to an *enhancement* of root growth (Figure 21.10), primarily by increasing the size of the root api-

FIGURE 21.8 Tobacco plants overexpressing the gene for cytokinin oxidase. The plant on the left is wild type. The two plants on the right are overexpressing two different constructs of the *Arabidopsis* gene for cytokinin oxidase: *AtCKX1* and *AtCKX2*. Shoot growth is strongly inhibited in the transgenic plants. (From Werner et al. 2001.)

cal meristem (Figure 21.11). Since the root is a major source of cytokinin, this result may indicate that cytokinins play opposite roles in regulating cell proliferation in root and shoot meristems.

An additional line of evidence linking cytokinin to the regulation of cell division in vivo came from analyses of

mutations in the cytokinin receptor (which will be discussed later in the chapter). Mutations in the cytokinin receptor disrupt the development of the root vasculature. Known as *cre1*, these mutants have no phloem in their roots; the root vascular system is composed almost entirely of xylem (see Chapters 4 and 10).

Further analysis revealed that this defect was due to an insufficient number of vasculature stem cells. That is, at the time of differentiation of the phloem and xylem, the pool of stem cells is abnormally small in *cre1* mutants; all the cells become committed to a xylem fate, and no stem cells remain to specify phloem. These results indicate that cytokinin plays a key role in regulating proliferation of the vasculature stem cells of the root.

Cytokinins Regulate Specific Components of the Cell Cycle

Cytokinins regulate cell division by affecting the controls that govern the passage of the cell through the cell division cycle. Zeatin levels were found to peak in synchronized culture tobacco cells at the end of S phase, mitosis, and G_1 phase.

Cytokinins were discovered in relation to their ability to stimulate cell division in tissues supplied with an optimal level of auxin. Evidence suggests that both auxin and cytokinins participate in regulation of the cell cycle and that they do so by controlling the activity of cyclin-dependent kinases. As discussed in Chapter 1, *cyclin-dependent protein kinases* (CDKs), in concert with their regulatory subunits, the *cyclins*, are enzymes that regulate the eukaryotic cell cycle.

The expression of the gene that encodes the major CDK, Cdc2 (cell division cycle 2), is regulated by auxin (see Chapter 19). In pea root tissues, *CDC2* mRNA was induced within 10 minutes after treatment with auxin, and high levels of CDK are induced in tobacco pith when it is cultured on medium containing auxin (John et al. 1993). However, the CDK induced by auxin is enzymatically inactive, and
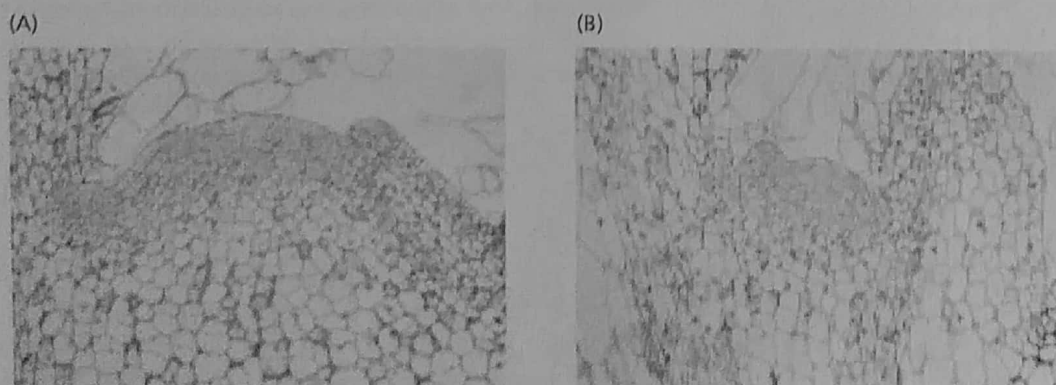
(A)   (B)

FIGURE 21.9 Cytokinin is required for normal growth of the shoot apical meristem. (A) Longitudinal section through the shoot apical meristem of a wild-type tobacco plant. (B) Longitudinal section through the shoot apical meristem of a transgenic tobacco overexpressing the gene that encodes cytokinin oxidase (*AtCKX1*). Note the reduction in the size of the apical meristem in the cytokinin-deficient plant. (From Werner et al. 2001.)

FIGURE 21.10 Cytokinin suppresses the growth of roots. The cytokinin-deficient *AtCKX1* roots (right) are larger than those of the wild-type tobacco plant (left). (From Werner et al. 2001.)
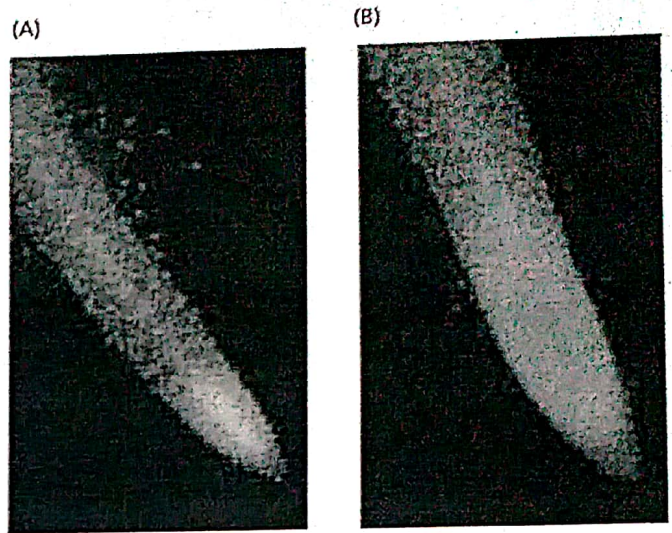

FIGURE 21.11 Cytokinin suppresses the size and cell division activity of roots. (A) Wild type. (B) *AtCKX1*. These roots were stained with the fluorescent dye, 4′, 6-diamidino-2-phenylindole, which stains the nucleus. (From Werner et al. 2001.)

high levels of CDK alone are not sufficient to permit cells to divide.

Cytokinin has been linked to the activation of a Cdc25-like phosphatase, whose role is to remove an inhibitory phosphate group from the Cdc2 kinase (Zhang et al. 1996). This action of cytokinin provides one potential link between cytokinin and auxin in regulating the cell cycle.

Recently, a second major input for cytokinin in regulating the cell cycle has emerged. Cytokinins elevate the expression of the *CYCD3* gene, which encodes a *D-type cyclin* (Soni et al. 1995; Riou-Khamlichi et al. 1999). In animal cells, D-type cyclins are regulated by a wide variety of growth factors and play a key role in regulating the passage through the restriction point of the cell cycle in G_1. D-type cyclins are thus key players in the regulation of cell proliferation.

In *Arabidopsis*, *CYCD3* is expressed in proliferating tissues such as shoot meristems and young leaf primordia. In a crucial experiment, it was found that overexpression of *CYCD3* can bypass the cytokinin requirement for cell proliferation in culture (Figure 21.12) (Riou-Khamlichi et al. 1999). These and other results suggest that a major mechanism for cytokinin's ability to stimulate cell division is its increase of *CYCD3* function.

The Auxin:Cytokinin Ratio Regulates Morphogenesis in Cultured Tissues

Shortly after the discovery of kinetin, it was observed that the differentiation of cultured callus tissue derived from tobacco pith segments into either roots or shoots depends on the ratio of auxin to cytokinin in the culture medium. Whereas high auxin:cytokinin ratios stimulated the formation of roots, low auxin:cytokinin ratios led to the formation of shoots. At intermediate levels the tissue grew as an undifferentiated callus (Figure 21.13) (Skoog and Miller 1965).

The effect of auxin:cytokinin ratios on morphogenesis can also be seen in crown gall tumors by mutation of the T-DNA of the *Agrobacterium* Ti plasmid (Garfinkel et al. 1981). Mutating the *ipt* gene (the *tmr* locus) of the Ti plasmid blocks zeatin biosynthesis in the infected cells. The resulting high auxin:cytokinin ratio in the tumor cells causes the proliferation of roots instead of undifferentiated callus tissue. In contrast, mutating either of the genes for auxin biosynthesis (*tms* locus) low-

FIGURE 21.12 *CYCD3*-expressing callus cells can divide in the absence of cytokinin. Leaf explants from transgenic Arabidopsis plants expressing *CYCD3* under a cauliflower mosaic virus 35S promoter were induced to form calluses through culturing in the presence of auxin plus cytokinin or auxin alone. The wild-type control calluses required cytokinin to grow. The *CYCD3*-expressing calluses grew well on medium containing auxin alone. The photographs were taken after 29 days. (From Riou-Khamlichi et al. 1999.)
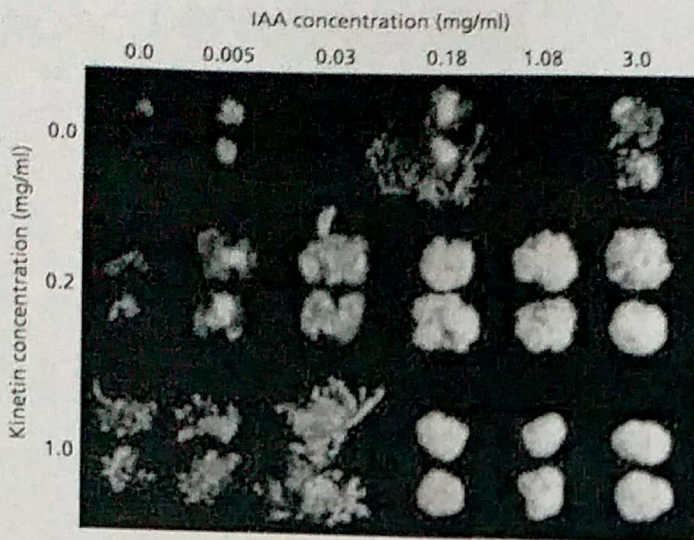

Auxin + cytokinin

wild type

CYCD3 overexpressor

Auxin

wild type

CYCD3 overexpressor
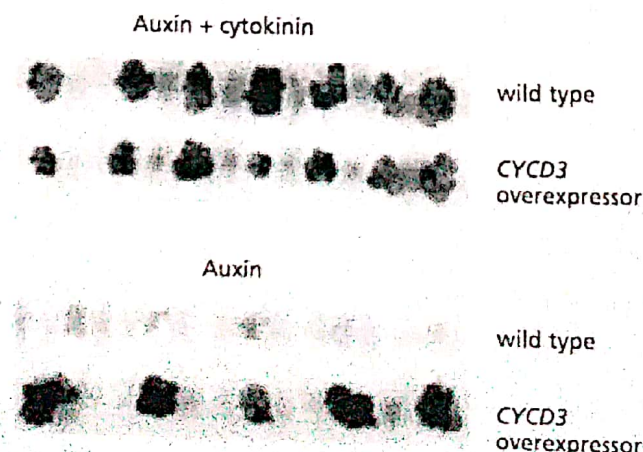
FIGURE 21.13 The regulation of growth and organ formation in cultured tobacco callus at different concentrations of auxin and kinetin. At low auxin and high kinetin concentrations (lower left) buds developed. At high auxin and low kinetin concentrations (upper right) roots developed. At intermediate or high concentrations of both hormones (middle and lower right) undifferentiated callus developed. (Courtesy of Donald Armstrong.)

ers the auxin:cytokinin ratio and stimulates the proliferation of shoots (Figure 21.14) (Akiyoshi et al. 1983). These partially differentiated tumors are known as teratomas.

Cytokinins Modify Apical Dominance and Promote Lateral Bud Growth

One of the primary determinants of plant form is the degree of apical dominance (see Chapter 19). Plants with strong apical dominance, such as maize, have a single growing axis with few lateral branches. In contrast, many lateral buds initiate growth in shrubby plants.

Although apical dominance may be determined primarily by auxin, physiological studies indicate that cytokinins play a role in initiating the growth of lateral buds. For example, direct applications of cytokinins to the axillary buds of many species stimulate cell division activity and growth of the buds.

The phenotypes of cytokinin-overproducing mutants are consistent with this result. Wild-type tobacco shows strong apical dominance during vegetative development, and the lateral buds of cytokinin overproducers grow vigorously, developing into shoots that compete with the main shoot. Consequently, cytokinin-overproducing plants tend to be bushy.
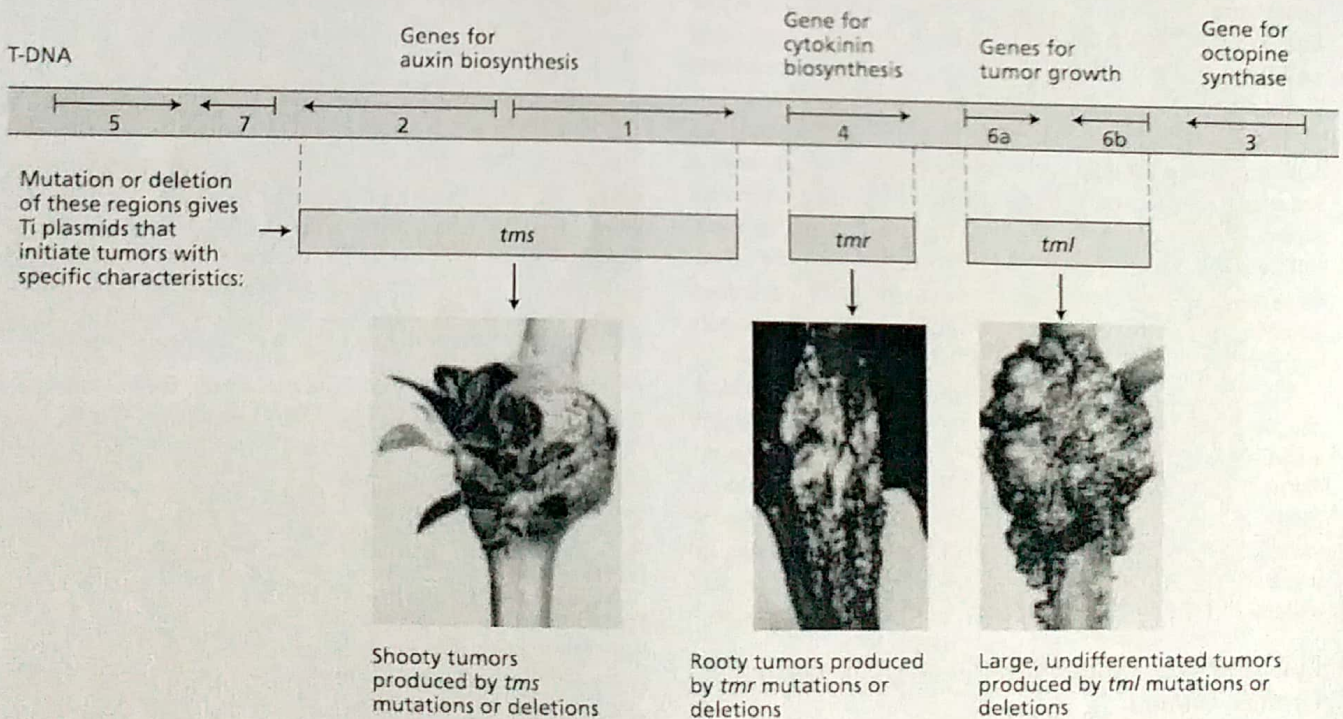

FIGURE 21.14 Map of the T-DNA from an *Agrobacterium* Ti plasmid, showing the effects of T-DNA mutations on crown gall tumor morphology. Genes 1 and 2 encode the two enzymes involved in auxin biosynthesis; gene 4 encodes a cytokinin biosynthesis enzyme. Mutations in these genes produce the phenotypes illustrated. (From Morris 1986, courtesy of R. Morris.)
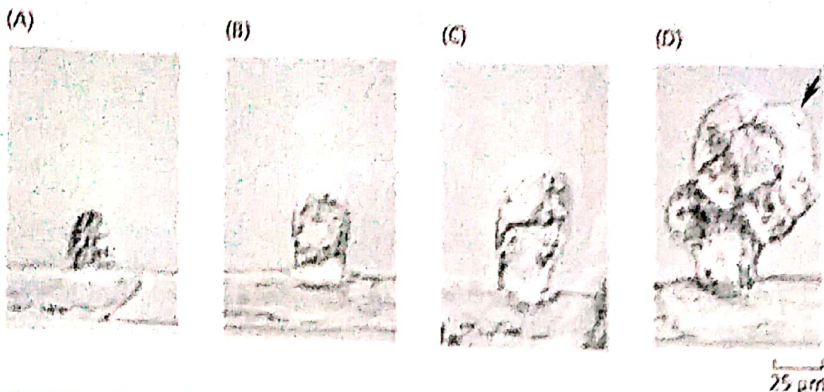
(A) (B) (C) (D)

25 μm

FIGURE 21.15 Bud formation in the moss *Funaria* begins with the formation of a protuberance at the apical ends of certain cells in the protonema filament. A–D show various stages of bud development. Once formed, the bud goes on to produce the leafy gametophyte stage of the moss. (Courtesy of K. S. Schumaker.)

Cytokinins Induce Bud Formation in a Moss

Thus far we have restricted our discussion of plant hormones to the angiosperms. However, many plant hormones are present and developmentally active in representative species throughout the plant kingdom. The moss *Funaria hygrometrica* is a well-studied example. The germination of moss spores gives rise to a filament of cells called a *protonema* (plural *protonemata*). The protonema elongates and undergoes cell divisions at the tip, and it forms branches some distance back from the tip (see Web Essay 21.1).

The transition from filamentous growth to leafy growth begins with the formation of a swelling or protuberance near the apical ends of specific cells (Figure 21.15). An asymmetric cell division follows, creating the initial cell. The initial cell then divides mitotically to produce the bud, the structure that gives rise to the leafy gametophyte. During normal growth, buds and branches are regularly initiated, usually beginning at the third cell from the tip of the filament.

Light, especially red light, is required for bud formation in *Funaria*. In the dark, buds fail to develop, but cytokinin added to the medium can substitute for the light requirement. Cytokinin not only stimulates normal bud development; it also increases the total number of buds (Figure 21.16). Even very low levels of cytokinin (picomolar, or 10^{-12} M) can stimulate the first step in bud formation: the swelling at the apical end of the specific protonemal cell.

Cytokinin Overproduction Has Been Implicated in Genetic Tumors

Many species in the genus *Nicotiana* can be crossed to generate interspecific hybrids. More than 300 such interspecific hybrids have been produced; 90% of these hybrids are normal, exhibiting phenotypic characteristics intermediate between those of both parents. The plant used for cigarette tobacco, *Nicotiana tabacum*, for example, is an interspecific hybrid. However, about 10% of these interspecific crosses result in progeny that tend to form spontaneous tumors

called genetic tumors (Figure 21.17) (Smith 1988).

Genetic tumors are similar morphologically to those induced by *Agrobacterium tumefaciens*, discussed at the beginning of this chapter, but genetic tumors form spontaneously in the absence of any external inducing agent. The tumors are composed of masses of rapidly proliferating cells in regions of the plant that ordinarily would contain few dividing cells. Furthermore, the cells divide without differentiating into the cell types normally associated with the tissues giving rise to the tumor.

Nicotiana hybrids that produce genetic tumors have abnormally high levels of both auxin and cytokinins. Typically, the cytokinin
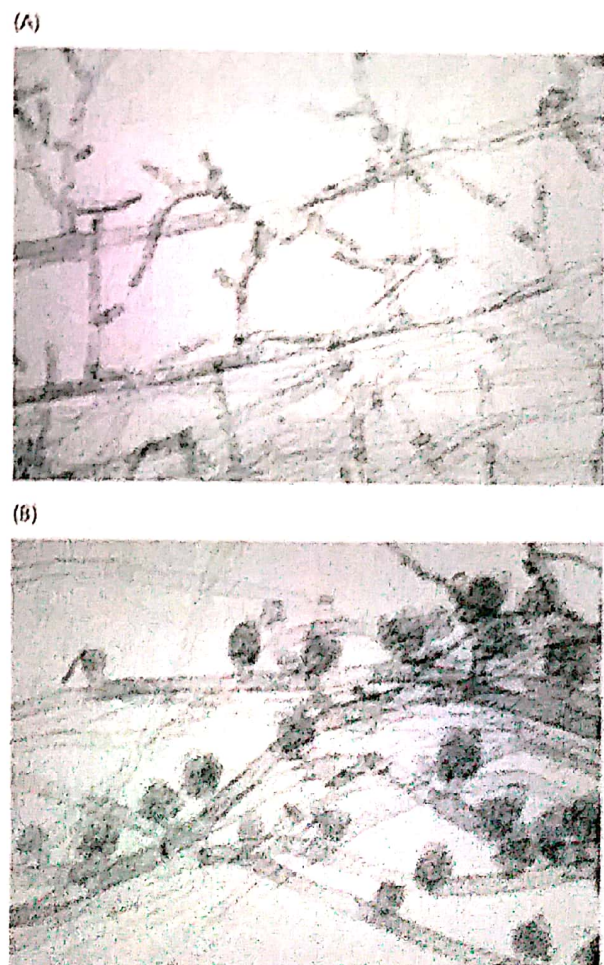
(A)

(B)

FIGURE 21.16 Cytokinin stimulates bud development in *Funaria*. (A) Control protonemal filaments. (B) Protonemal filaments treated with benzyladenine. (Courtesy of H. Kende.)
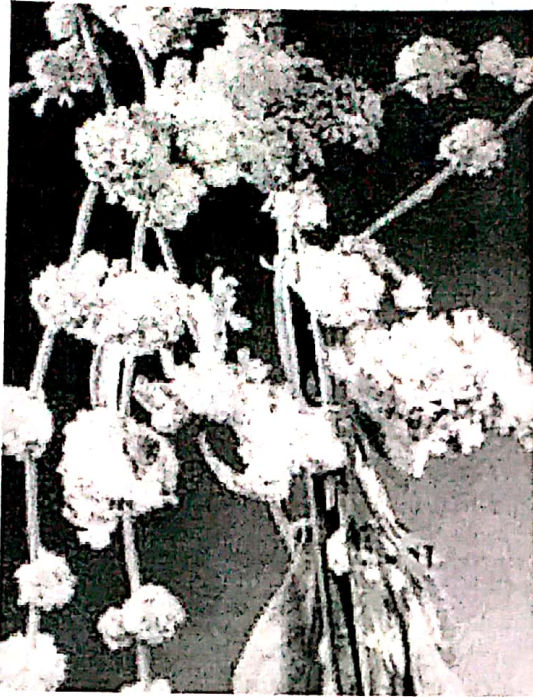
FIGURE 21.17   Expression of genetic tumors in the hybrid *Nicotiana langsdorffii* × *N. glauca*. (From Smith 1988.)

levels in tumor-prone hybrids are five to six times higher than those found in either parent.

Cytokinins Delay Leaf Senescence

Leaves detached from the plant slowly lose chlorophyll, RNA, lipids, and protein, even if they are kept moist and provided with minerals. This programmed aging process leading to death is termed **senescence** (see Chapters 16 and 23). Leaf senescence is more rapid in the dark than in the light. Treating isolated leaves of many species with cytokinins will delay their senescence.

Although applied cytokinins do not prevent senescence completely, their effects can be dramatic, particularly when the cytokinin is sprayed directly on the intact plant. If only one leaf is treated, it remains green after other leaves of similar developmental age have yellowed and dropped off the plant. Even a small spot on a leaf will remain green if treated with a cytokinin, after the surrounding tissues on the same leaf begin to senesce.

Unlike young leaves, mature leaves produce little if any cytokinin. Mature leaves may depend on root-derived cytokinins to postpone their senescence. Senescence is initiated in soybean leaves by seed maturation—a phenomenon known as *monocarpic senescence*—and can be delayed by seed removal. Although the seedpods control the onset of senescence, they do so by controlling the delivery of root-derived cytokinins to the leaves.

The cytokinins involved in delaying senescence are primarily zeatin riboside and dihydrozeatin riboside, which may be transported into the leaves from the roots through the xylem, along with the transpiration stream (Noodén et al. 1990).

To test the role of cytokinin in regulating the onset of leaf senescence, tobacco plants were transformed with a chimeric gene in which a senescence-specific promoter was used to drive the expression of the *ipt* gene (Gan and Amasino 1995). The transformed plants had wild-type levels of cytokinins and developed normally, up to the onset of leaf senescence.

As the leaves aged, however, the senescence-specific promoter was activated, triggering the expression of the *ipt* gene within leaf cells just as senescence would have been initiated. The resulting elevated cytokinin levels not only blocked senescence, but also limited further expression of the *ipt* gene, preventing cytokinin overproduction (Figure 21.18). This result suggests that cytokinins are a natural regulator of leaf senescence.

Plant expressing *ipt* gene remains green and photosynthetic

Age-matched control: advanced senescence, no photosynthesis

FIGURE 21.18   Leaf senescence is retarded in a transgenic tobacco plant containing a cytokinin biosynthesis gene, *ipt*. The *ipt* gene is expressed in response to signals that induce senescence. (From Gan and Amasino 1995, courtesy of R. Amasino.)

Cytokinins Promote Movement of Nutrients

Cytokinins influence the movement of nutrients into leaves from other parts of the plant, a phenomenon known as *cytokinin-induced nutrient mobilization*. This process is revealed when nutrients (sugars, amino acids, and so on) radiolabeled with ^{14}C or ^{3}H are fed to plants after one leaf or part of a leaf is treated with a cytokinin. Later the whole plant is subjected to autoradiography to reveal the pattern of movement and the sites at which the labeled nutrients accumulate.

Experiments of this nature have demonstrated that nutrients are preferentially transported to, and accumulated in, the cytokinin-treated tissues. It has been postulated that the hormone causes nutrient mobilization by creating a new source–sink relationship. As discussed in Chapter 10, nutrients translocated in the phloem move from a site of production or storage (the source) to a site of utilization (the sink). The metabolism of the treated area may be stimulated by the hormone so that nutrients move toward it. However, it is not necessary for the nutrient itself to be metabolized in the sink cells because even nonmetabolizable substrate analogs are mobilized by cytokinins (Figure 21.19).

Cytokinins Promote Chloroplast Development

Although seeds can germinate in the dark, the morphology of dark-grown seedlings is very different from that of light-grown seedlings (see Chapter 17): Dark-grown seedlings are said to be **etiolated**. The hypocotyl and internodes of etiolated seedlings are more elongated, cotyledons and leaves do not expand, and chloroplasts do not mature. Instead of maturing as chloroplasts, the proplastids of dark-grown seedlings develop into **etioplasts**, which do not synthesize chlorophyll or most of the enzymes and structural proteins required for the formation of the chloroplast thylakoid system and photosynthesis machinery. When seedlings germinate in the light, chloroplasts mature directly from the proplastids present in the embryo, but etioplasts also can mature into chloroplasts when etiolated seedlings are illuminated.

If the etiolated leaves are treated with cytokinin before being illuminated, they form chloroplasts with more extensive grana, and chlorophyll and photosynthetic enzymes are synthesized at a greater rate upon illumination (Figure 21.20). These results suggest that cytokinins—along with other factors, such as light, nutrition, and development—regulate the synthesis of photosynthetic pigments and proteins. The ability of exogenous cytokinin to enhance de-etiolation of dark-grown seedlings is mimicked by certain mutations that lead to cytokinin overproduction. (For more on how cytokinins promote light-mediated development, see Web Topic 21.7.)

Cytokinins Promote Cell Expansion in Leaves and Cotyledons

The promotion of cell enlargement by cytokinins is most clearly demonstrated in the cotyledons of dicots with leafy cotyledons, such as mustard, cucumber, and sunflower. The cotyledons of these species expand as a result of cell enlargement during seedling growth. Cytokinin treatment promotes additional cell expansion, with no increase in the dry weight of the treated cotyledons.

Leafy cotyledons expand to a much greater extent when the seedlings are grown in the light than in the dark, and cytokinins promote cotyledon growth in both light- and dark-grown seedlings (Figure 21.21). As with auxin-

---

In seedling A, the left cotyledon was sprayed with water as a control. The left cotyledon of seedling B, and the right cotyledon of seedling C, were each sprayed with a solution containing 50mM kinetin.

The dark stippling represents the distribution of the radioactive amino acid as revealed by autoradiography.

The results show that the cytokinin-treated cotyledon has become a nutrient sink. However, radioactivity is retained in the cotyledon to which the amino acid was applied when the labeled cotyledon is treated with kinetin (seedling C).
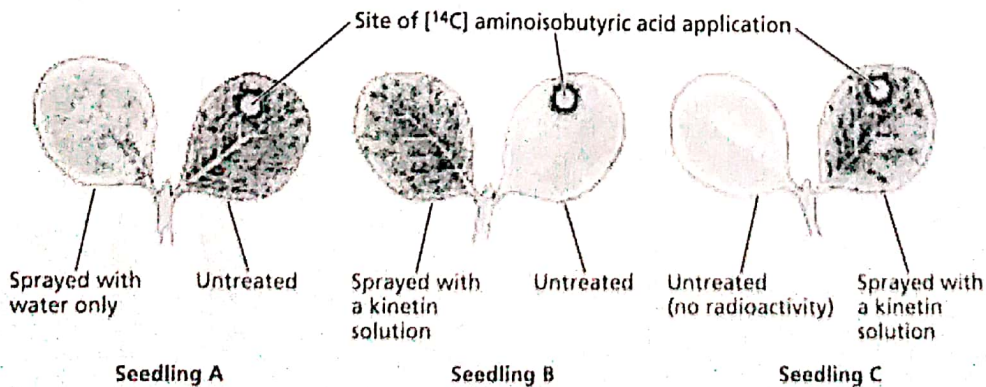

Site of [^{14}C] aminoisobutyric acid application

| Sprayed with water only | Untreated | Sprayed with a kinetin solution | Untreated | Untreated (no radioactivity) | Sprayed with a kinetin solution |

Seedling A   Seedling B   Seedling C

FIGURE 21.19 The effect of cytokinin on the movement of an amino acid in cucumber seedlings. A radioactively labeled amino acid that cannot be metabolized, such as aminoisobutyric acid, was applied as a discrete spot on the right cotyledon of each of these seedlings. (Drawn from data obtained by K. Mothes.)

(A)

(B)

FIGURE 21.20 Cytokinin influence on the development of wild-type *Arabidopsis* seedlings grown in darkness. (A) Plastids develop as etioplasts in the untreated, dark-grown control. (B) Cytokinin treatment resulted in thylakoid formation in the plastids of dark-grown seedlings. (From Chory et al. 1994, courtesy of J. Chory, © American Society of Plant Biologists, reprinted with permission.)
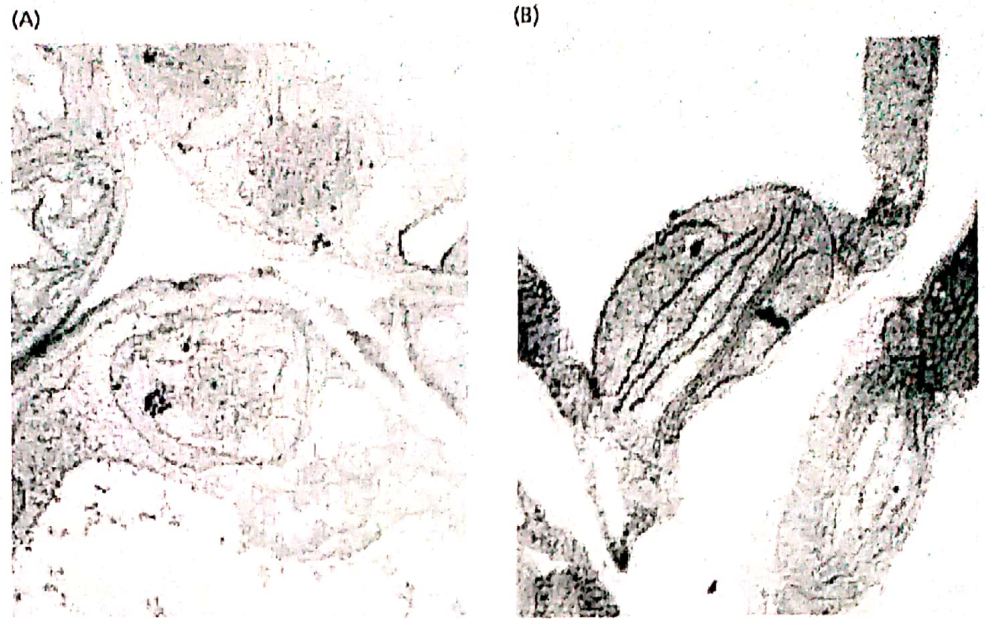
induced growth, cytokinin-stimulated expansion of radish cotyledons is associated with an increase in the mechanical extensibility of the cell walls. However, cytokinin-induced wall loosening is not accompanied by proton extrusion. Neither auxin nor gibberellin promotes cell expansion in cotyledons.
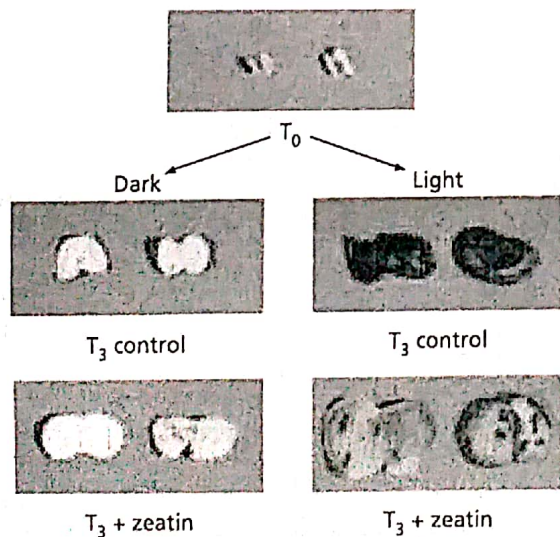

FIGURE 21.21 The effect of cytokinin on the expansion of radish cotyledons. The experiment described here shows that the effects of light and cytokinin are additive. T_0 represents germinating radish seedlings before the experiment began. The detached cotyledons were incubated for 3 days (T_3) in either darkness or light with or without 2.5 mM zeatin. In both the light and the dark, zeatin-treated cotyledons expanded more than in the control. (From Huff and Ross 1975.)

Cytokinins Regulate Growth of Stems and Roots

Although endogenous cytokinins are clearly required for normal cell proliferation in the apical meristem, and therefore normal shoot growth (see Figure 21.9), applied cytokinins typically inhibit the process of cell elongation in both stems and roots. For example, exogenous cytokinin inhibits hypocotyl elongation at concentrations that promote leaf and cotyledon expansion in the dark-grown seedlings.

In related experiments, internode and root elongation are both inhibited in transgenic plants expressing the *ipt* gene and in cytokinin-overproducing mutants. It is likely that the inhibition of hypocotyl and internode elongation induced by excess cytokinin is due to the production of ethylene, and this inhibition thus may represent another example of the interdependence of hormonal regulatory pathways (Cary et al. 1995; Vogel et al. 1998).

On the other hand, other experiments suggest that endogenous cytokinins at normal physiological concentrations inhibit root growth. For example, a weak allele of a cytokinin receptor mutant and a loss-of-function allele of a cytokinin signaling element both have longer roots than the wild type (Inoue et al. 2001; Sakai et al. 2001). As previously noted, transgenic tobacco engineered to overexpress cytokinin oxidase (and thus to have lower levels of cytokinin) also has longer roots than its wild-type counterpart (see Figure 21.10) (Werner et al. 2001). These results indicate that endogenous cytokinins may negatively regulate root elongation.

Cytokinin-Regulated Processes Are Revealed in Plants That Overproduce Cytokinin

The *ipt* gene from the *Agrobacterium* Ti plasmid has been introduced into many species of plants, resulting in

cytokinin overproduction. These transgenic plants exhibit an array of developmental abnormalities that tell us a great deal about the biological role of cytokinins.

As discussed earlier, plant tissues transformed by *Agrobacterium* carrying a wild-type Ti plasmid proliferate as tumors as a result of the overproduction of both auxin and cytokinin. And as mentioned already, if all of the other genes in the T-DNA are deleted and plant tissues are transformed with T-DNA containing only a selective antibiotic resistance marker gene and the *ipt* gene, shoots proliferate instead of callus.

The shoot teratomas formed by *ipt*-transformed tissues are difficult to root, and when roots are formed, they tend to be stunted in their growth. As a result, it is difficult to obtain plants from shoots expressing the *ipt* gene under the control of its own promoter because the promoter is a constitutive promoter and the gene is continuously expressed.

To circumvent this problem, a variety of promoters whose expression can be regulated have been used to drive the expression of the *ipt* gene in the transformed tissues. For example, several studies have employed a heat shock promoter, which is induced in response to elevated temperature, to drive inducible expression of the *ipt* gene in transgenic tobacco and *Arabidopsis*. In these plants, heat induction substantially increased the level of zeatin, zeatin riboside and ribotide, and *N*-conjugated zeatin.

These cytokinin-overproducing plants exhibit several characteristics that point to roles played by cytokinin in plant physiology and development:

- The shoot apical meristems of cytokinin-overproducing plants produce more leaves.

- The leaves have higher chlorophyll levels and are much greener.

- Adventitious shoots may form from unwounded leaf veins and petioles.

- Leaf senescence is retarded.

- Apical dominance is greatly reduced.

- The more extreme cytokinin-overproducing plants are stunted, with greatly shortened internodes.

- Rooting of stem cuttings is reduced, as is the root growth rate.

Some of the consequences of cytokinin overproduction could be highly beneficial for agriculture if synthesis of the hormone can be controlled. Because leaf senescence is delayed in the cytokinin-overproducing plants, it should be possible to extend their photosynthetic productivity (which we'll discuss shortly).

In addition, cytokinin production could be linked to damage caused by predators. For example, tobacco plants transformed with an *ipt* gene under the control of the promoter from a wound-inducible protease inhibitor II gene were more resistant to insect damage. The tobacco hornworm consumed up to 70% fewer tobacco leaves in plants that expressed the *ipt* gene driven by the protease inhibitor promoter (Smigocki et al. 1993).

CELLULAR AND MOLECULAR MODES OF CYTOKININ ACTION

The diversity of the effects of cytokinin on plant growth and development is consistent with the involvement of signal transduction pathways with branches leading to specific responses. Although our knowledge of how cytokinin works at the cellular and molecular levels is still quite fragmentary, significant progress has been achieved. In this section we will discuss the nature of the cytokinin receptor and various cytokinin-regulated genes, as well as a model for cytokinin signaling based on current information.

A Cytokinin Receptor Related to Bacterial Two-Component Receptors Has Been Identified

The first clue to the nature of the cytokinin receptor came from the discovery of the *CKI1* gene. *CKI1* was identified in a screen for genes that, when overexpressed, conferred cytokinin-independent growth on *Arabidopsis* cells in culture. As discussed already, plant cells generally require cytokinin in order to divide in culture. However, a cell line that overexpresses *CKI1* is capable of growing in culture in the absence of added cytokinin.

CKI1 encodes a protein similar in sequence to bacterial two-component sensor histidine kinases, which are ubiquitous receptors in prokaryotes (see Chapter 14 on the web site and Chapter 17). Bacterial two-component regulatory systems mediate a range of responses to environmental stimuli, such as osmoregulation and chemotaxis. Typically these systems are composed of two functional elements: a *sensor histidine kinase*, to which a signal binds, and a downstream *response regulator*, whose activity is regulated via phosphorylation by the sensor histidine kinase. The sensor histidine kinase is usually a membrane-bound protein that contains two distinct domains, called the input and histidine kinase, or "transmitter," domains (Figure 21.22).

Detection of a signal by the input domain alters the activity of the histidine kinase domain. Active sensor kinases are dimers that transphosphorylate a conserved histidine residue. This phosphate is then transferred to a conserved aspartate residue in the receiver domain of a cognate response regulator (see Figure 21.22), and this phosphorylation alters the activity of the kinases. Most response regulators also contain *output* domains that act as transcription factors.

The phenotype resulting from *CKI1* overexpression, combined with its similarity to bacterial receptors, suggested that the CKI1 and/or similar histidine kinases are cytokinin receptors. Support for this model came from identification of the *CRE1* gene (Inoue et al. 2001).

Simple two-component signaling system

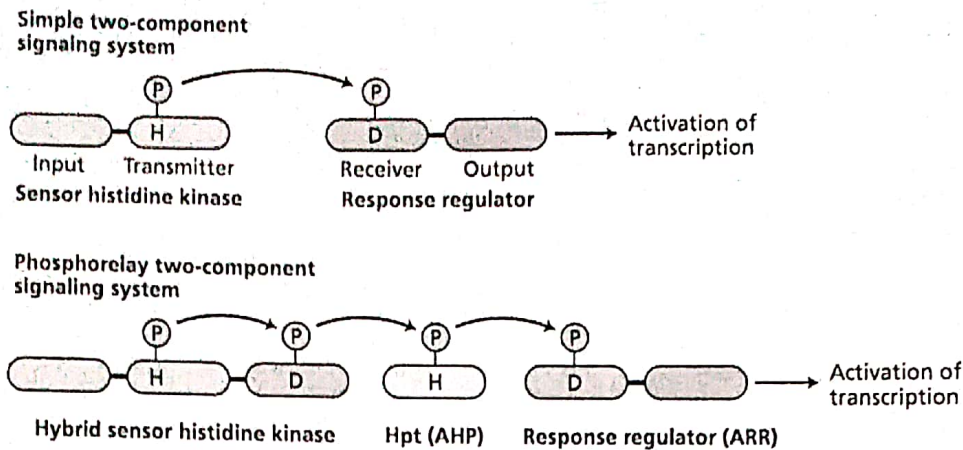**Phosphorelay two-component signaling system**

FIGURE 21.22 Simple versus phosphorelay types of two-component signaling systems. (A) In simple two-component systems, the input domain is the site where the signal is sensed. This regulates the activity of the histidine kinase domain, which when activated autophosphorylates on a conserved His residue. The phosphate is then transferred to an Asp residue that resides within the receiver domain of a response regulator. Phosphorylation of this Asp regulates the activity of the output domain of the response regulator, which in many cases is a transcription factor. (B) In the phosphorelay-type two-component signaling system, an extra set of phosphotransfers is mediated by a histidine phosphotransfer protein (Hpt), called AHP in *Arabidopsis*. The *Arabidopsis* response regulators are called ARRs. H = histidine, D = aspartate.

Like *CKI1*, *CRE1* encodes a protein similar to bacterial histidine kinases. Loss-of-function *cre1* mutations were identified in a genetic screen for mutants that failed to develop shoots from undifferentiated tissue culture cells in response to cytokinin. This is essentially the opposite screen from the one just described, from which the *CKI1* gene was identified by a gain-of-function (ability to divide in the absence of cytokinin) mutation. The *cre1* mutants are also resistant to the inhibition of root elongation observed in response to cytokinin.

Convincing evidence that *CRE1* encodes a cytokinin receptor came from analysis of the expression of the protein in yeast. Yeast cells also contain a sensor histidine kinase, and deletion of the gene that encodes this kinase—*SLN1*—is lethal. Expression of *CRE1* in *SLN1*-deficient yeast can restore viability, *but only if cytokinins are present in the medium*. Thus the activity of CRE1 (i.e., its ability to replace SLN1) is dependent on cytokinin, which, coupled with the cytokinin-insensitive phenotype of the *cre1* mutants in *Arabidopsis*, unequivocally demonstrates that CRE1 is a cytokinin receptor. It remains to be determined if CKI1 is also a cytokinin receptor.

Two other genes in the *Arabidopsis* genome (*AHK2* and *AHK3*) are closely related to *CRE1*, suggesting that, like the ethylene receptors (see Chapter 22), the cytokinin receptors are encoded by a multigene family. Indeed, it has been demonstrated that cytokinins bind to the predicted extracellular domains of CRE1, AHK2, and AHK3 with high affinity, confirming that they are indeed cytokinin receptors (Yamada et al. 2001). This raises the possibility that these genes are at least partially genetically redundant (as are the ethylene receptors), which may explain the relatively mild phenotypes that result from loss-of-function *cre1* mutations.

Cytokinins Cause a Rapid Increase in the Expression of Response Regulator Genes

One of the primary effects of cytokinin is to alter the expression of various genes. The first set of genes to be upregulated in response to cytokinin are the *ARR* (*Arabidopsis* response regulator) genes. These genes are homologous to the receiver domain of bacterial two-component response regulators, the downstream target of sensor histidine kinases (see the previous section).

In *Arabidopsis*, response regulators are encoded by a multigene family. They fall into two basic classes: the **type-A ARR** genes, which are made up solely of a receiver domain, and the **type-B ARR** genes, which contain a transcription factor domain in addition to the receiver domain (Figure 21.23). The rate of transcription of the type-A gene is increased within 10 minutes in response to applied cytokinin (Figure 21.24) (D'Agostino et al. 2000). This rapid induction is specific for cytokinin and does not require new protein synthesis. Both of these features are hallmarks of primary response genes (discussed in Chapters 17 and 19).

The rapid induction of the type-A genes, coupled with their similarity to signaling elements predicted to act downstream of sensor histidine kinases, suggests that these elements act downstream of the CRE1 cytokinin receptor family to mediate the primary cytokinin response. Interestingly, one of these type-A genes, *ARR5*, is expressed primarily in the apical meristems of both shoots and roots (Figure 21.25), consistent with a role in regulating cell proliferation, a key aspect of cytokinin action.

FIGURE 21.23 Phylogenetic tree of *Arabidopsis* response regulators. The top part of the figure shows a phylogenetic tree that represents the degree of relatedness of the receiver domains present in the *Arabidopsis* genome. The closer two proteins are on the tree, the more similar are their amino acid sequences. Note that these proteins fall into two distinct groups, or clades, called the type-A ARRs (blue) and the type-B ARRs (red). These differences in sequence are also reflected in a distinct domain structure, as depicted below the tree. The type-A ARRs consist solely of a receiver domain, but the type-A proteins also contain a fused output domain at the carboxy-terminus.

Histidine Phosphotransferases May Mediate the Cytokinin Signaling Cascade
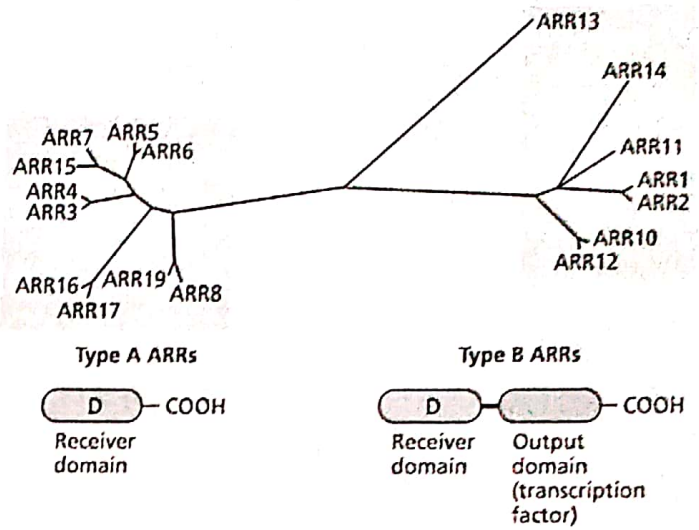
The expression of a wide variety of other genes is altered in response to cytokinin, but generally with slower kinetics than the type-A genes. These include the gene that encodes nitrate reductase, light-regulated genes such as *LHCB* and *SSU*, and defense-related genes such as *PR1*, as well as genes that encode an extensin (cell wall protein rich in hydroxyproline), rRNAs, cytochrome P450s, and peroxidase. Cytokinin elevates the expression of these genes both by increasing the rate of transcription (as in the case of the type-A *ARRs*) and/or by a stabilization of the RNA transcript (e.g., the extensin gene).

From the preceding discussions we have seen that cytokinin binds to the CRE1 receptors to initiate a response that culminates in the elevation of transcription of the type-A ARRs. The type-A ARR proteins, in turn, may regulate the expression of numerous other genes, as well as the activities of various target proteins that ultimately alter cellular function. How is the signal propagated from CRE1 (which is at the plasma membrane) to the nucleus to alter type-A *ARR* transcription?

One set of genes that are likely to be involved in this signaling cascade encode the AHP (*Arabidopsis* histidine phosphotransfer) proteins. In two-component systems that involve a sensor kinase fused to a receiver domain (the structure of most eukaryotic sensor histidine kinases, including those of the CRE1 family), there is an additional set of phosphotransfers that are mediated by a histidine phosphotransfer protein (Hpt).

Phosphate is first transferred from ATP to a histidine within the histidine kinase domain, and then transferred to an aspartate residue on the fused receiver. From the aspartate residue the phosphate group is then transferred to a histidine on the Hpt protein and then finally to an aspartate on the receiver domain of the response regulator (see Figure 21.22). This phosphorylation of the receiver domain of the response regulator alters its activity. Thus, Hpt proteins are predicted to mediate the phosphotransfer between sensor kinases and response regulators.

In *Arabidopsis* there are 5 Hpt genes, called *AHPs*. The AHP proteins have been shown to physically associate with receiver
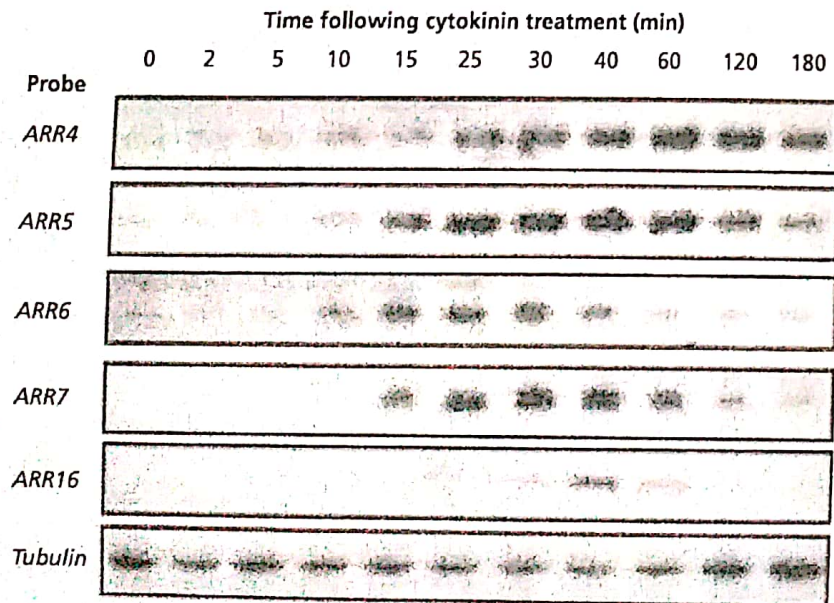
FIGURE 21.24 Induction of type-A *ARR* genes in response to cytokinin. RNA from *Arabidopsis* seedlings treated for the indicated time with cytokinin was isolated and analyzed by Northern blotting. Each row shows the result of probing the Northern blot with an individual type-A gene, and each lane contains RNA derived from *Arabidopsis* seedlings treated for the indicated time with cytokinin. The darker the band, the higher the level of *ARR* mRNA in that sample. (From D'Agostino et al. 2000.)

(A)   (B)   (C)
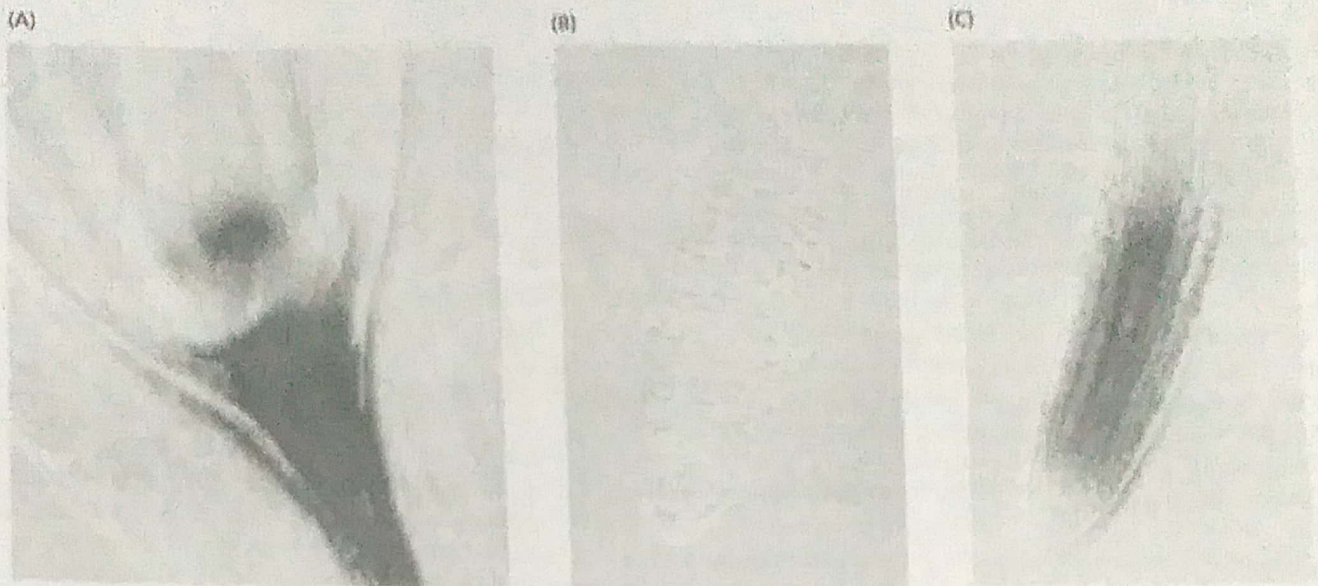

FIGURE 21.25 Expression of *ARR5*. The pattern of *ARR5* expression was examined by fusion of the promoter to a *GUS* reporter gene (A) or by whole-mount in situ hybridization (B and C). For the latter, the tissue is hybridized with labeled single-stranded *ARR5* RNA in either the sense orientation (B) or the antisense (C). The sense RNA is a negative control and reveals background, nonspecific staining. The antisense probe specifically hybridizes with the *ARR5* mRNA present in the tissue, thereby revealing its spatial distribution. With both methods, *ARR5* expression is observed primarily in the apical meristems. (From D'Agostino et al. 2000.)

domains from several *Arabidopsis* histidine kinases, including CRE1, and a subset of the AHPs have been demonstrated to transiently translocate from the cytoplasm to the nucleus in response to cytokinin (Figure 21.26) (Hwang and Sheen 2001). This finding suggests that the AHPs are the immediate downstream targets of the activated CRE receptors, and that these proteins transduce the cytokinin signal into the nucleus.

responsive reporter have provided a likely answer (Hwang and Sheen 2001; Sakai et al. 2001).

Disruption of *ARR1*, one of the type-B *ARR* genes, reduces the induction of the type-A *ARR* genes in response

Cytokinin-Induced Phosphorylation Activates Transcription Factors

The question now becomes, How do the activated AHPs, once in the nucleus, act to regulate gene transcription? Genetic studies in intact *Arabidopsis* plants and overexpression studies in isolated *Arabidopsis* protoplasts using a cytokinin

FIGURE 21.26 Cytokinin induces the transient movement of some AHP proteins into the nucleus. *Arabidopsis* protoplasts expressing various *AHP* genes fused to green fluorescent protein (GFP) as a reporter were treated with zeatin and monitored for 1.5 hours. *AHP1-GFP* and *AHP2-GFP* show nuclear localization after 30 minutes, but this localization is transient in the case of *AHP1-GFP*. Zeatin did not seem to affect the distribution of *AHP5-GFP*. (From Hwang and Sheen 2001.)
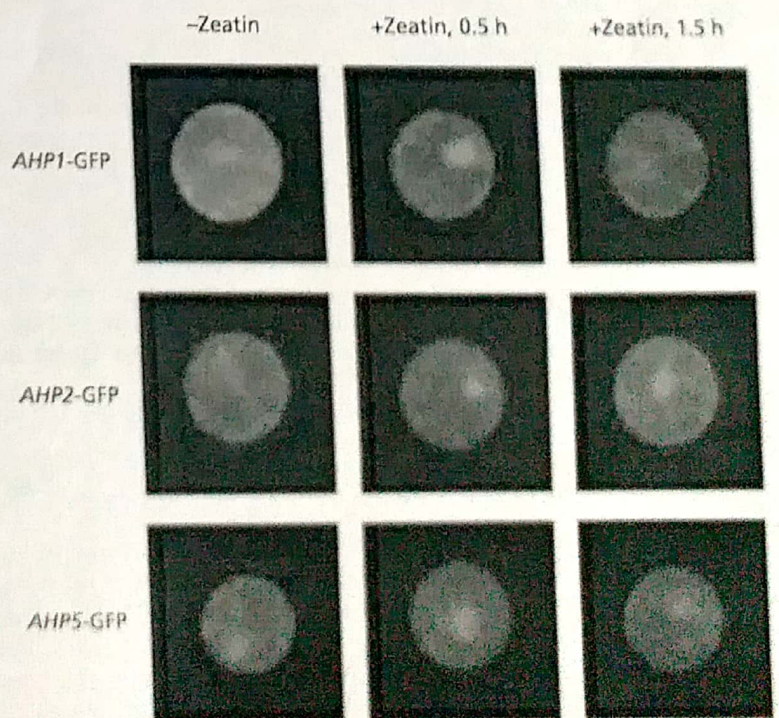
1. Cytokinin binds to CRE1, which is likely to occur as a dimer. Cytokinin binds to an extracellular portion of CRE1 called the CHASE domain. Two other hybrid sensor kinases (AHK2 and AHK3) containing a CHASE domain are also likely to act as cytokinin receptors in *Arabidopsis*.

2. Cytokinin binding to these receptors activates their histidine kinase activity. The phosphate is transferred to an asparate residue (D) on the fused receiver domains.

3. The phosphate is then transferred to a conserved histidine present in an AHP protein.

4. Phosphorylation causes the AHP protein to move into the nucleus, where it transfers the phosphate to an asparate residue located within the receiver domain of a type-B ARR.

5. The phosphorylation of the type-B ARR activates the output domain to induce transcription of genes encoding type-A ARRs.

6. The type-A ARRs are likely also to be phosphorylated by the AHP proteins.

7. The phosphorylated type-A ARRs interact with various effectors to mediate the changes in cell function appropriate to cytokinin (indicated in the model as "cytokinin responses").
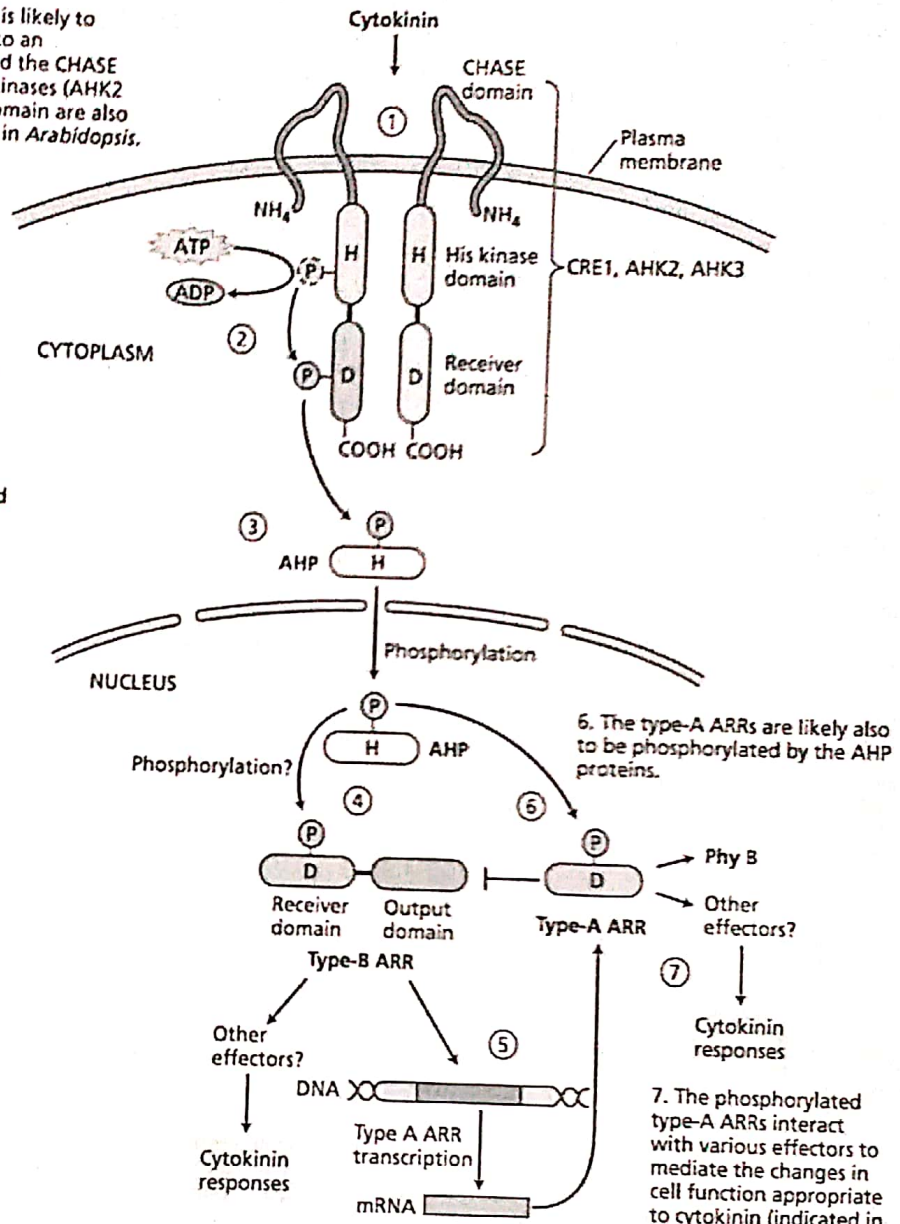
FIGURE 21.27 Model of cytokinin signaling. The near future should see significant refinement of this model, the tools are now in hand to analyze the interactions among these elements.

to cytokinin. Conversely, an increase in *ARR1* function increases the response of the type-A genes to cytokinin. This suggests that ARR1, which is a transcription factor, directly regulates transcription of the type-A *ARRs*, and that by analogy other members of the type-B *ARR* family (see Figure 21.23) also mediate cytokinin-regulated gene expression.

This conclusion is supported by the findings that type-B ARRs operate as transcriptional activators and that there are multiple binding sites for ARR1, a type-B ARR, in the 5′ DNA regulatory sequences of the type-A ARR genes.

A model of cytokinin signaling is presented in Figure 21.27. Cytokinin binds to the CRE1 receptor and initiates a phosphorylation cascade that results in the phosphorylation and activation of a subset of the type-B ARR proteins. Activation of the type-B proteins (transcription factors) leads to the transcriptional activation of the type-A genes. The type-A ARR proteins are likely also phosphorylated in response to cytokinin, and perhaps together with the type-B proteins, they interact with various targets to mediate the changes in cellular function, such as an activation of the cell

cycle. Type-A ARRs are also able to inhibit their own expression by an unknown mechanism, providing a negative feedback loop (see Figure 21.27). Much work needs to be done to confirm and refine this model, but we are beginning to glimpse for the first time the molecular basis for cytokinin action in plants.

SUMMARY

Mature plant cells generally do not divide in the intact plant, but they can be stimulated to divide by wounding, by infection with certain bacteria, and by plant hormones, including cytokinins. Cytokinins are N^6-substituted aminopurines that will initiate cell proliferation in many plant cells when they are cultured on a medium that also contains an auxin. The principal cytokinin of higher plants—zeatin, or *trans*-6-(4-hydroxy-3-methylbut-2-eny-lamino)purine—is also present in plants as a riboside or ribotide and as glycosides. These forms are generally also active as cytokinins in bioassays through their enzymatic conversion to the free zeatin base by plant tissue.

The first committed step in cytokinin biosynthesis—the transfer of the isopentenyl group from DMAPP to the 6 nitrogen of adenosine tri- and diphosphate—is catalyzed by isopentenyl transferase (IPT). The product of this reaction is readily converted to zeatin and other cytokinins. Cytokinins are synthesized in roots, in developing embryos, young leaves, fruits, and crown gall tissues. Cytokinins are also synthesized by plant-associated bacteria, insects, and nematodes.

Cytokinin oxidases degrade cytokinin irreversibly and may play a role in regulation of the levels of this hormone. Conjugation of both the side chain and the adenosine moiety to sugars (mostly glucose) also may play a role in the regulation of cytokinin levels and may target subpools of the hormone for distinct roles, such as transport. Cytokinins are also interconverted among the free base and the nucleoside and nucleotide forms.

Crown galls originate from plant tissues that have been infected with *Agrobacterium tumefaciens*. The bacterium injects a specific region of its Ti plasmid called T-DNA into wounded plant cells, and the T-DNA is incorporated into the host nuclear genome. The T-DNA contains a gene for cytokinin biosynthesis, as well as genes for auxin biosynthesis. These phyto-oncogenes are expressed in the plant cells, leading to hormone synthesis and unregulated proliferation of the cells to form the gall.

Cytokinins are most abundant in the young, rapidly dividing cells of the shoot and root apical meristems. They do not appear to be actively transported through living plant tissues. Instead, they are transported passively into the shoot from the root through the xylem, along with water and minerals. At least in pea, however, the shoot can regulate the flow of cytokinin from the root.

Cytokinins participate in the regulation of many plant processes, including cell division, morphogenesis of shoots and roots, chloroplast maturation, cell enlargement, and senescence. Both cytokinin and auxin regulate the plant cell cycle and are needed for cell division. The roles of cytokinins have been elucidated from application of exogenous cytokinins, the phenotype of transgenic plants designed to overexpress cytokinins as a result of introduction of the bacterial *ipt* gene, and recently from transgenic plants that have a reduced endogenous cytokinin content as a result of overexpression of cytokinin oxidase.

In addition to cell division, the ratio of auxin to cytokinin determines the differentiation of cultured plant tissues into either roots or buds: High ratios promote roots; low ratios, buds. Cytokinins also have been implicated in the release of axillary buds from apical dominance. In the moss *Funaria*, cytokinins greatly increase the number of "buds," the structures that give rise to the leafy gametophyte stage of development.

The mechanism of action of cytokinin is just beginning to emerge. A cytokinin receptor has been identified in *Arabidopsis*. This transmembrane protein is related to the bacterial two-component sensor histidine kinases. Cytokinins increase the abundance of several specific mRNAs. Some of these are primary response genes that are similar to bacterial two-component response regulators. The signal transduction mechanism from CRE1 to transcriptional activation of the type-A ARRs involves other homologs of two-component elements.

Web Material

Web Topics

21.1 Cultured Cells Can Acquire the Ability to Synthesize Cytokinins

The phenomenon of habituation is described, whereby callus tissues become cytokinin independent.

21.2 Structures of Some Naturally Occurring Cytokinins

The structures of various naturally occurring cytokinins are presented.

21.3 Various Methods Are Used to Detect and Identify Cytokinins

Cytokinins can be qualified using immunological and sensitive physical methods.

21.4 Cytokinins Are Also Present in Some tRNAs in Animal and Plant Cells

Modified adenosines near the 3′ end of the anticodons of some tRNAs have cytokinin activity.