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Cytokinins can be conjugated to N- and O-glucosides. While N-glucosylation generally leads to irretrievable loss of activity, O-glucosides are thought to play an important role in cytokinin storage and homeostasis since they can be hydrolyzed to the corresponding aglycones. The first zeatin O-glycosyltransferase genes isolated were *ZOG1* and *ZOX1* from beans (Martin et al., 1999a,b), and more recently cytokinin O- and N-glucosyltransferase genes were identified in Arabidopsis (Hou et al., 2005).

To study the effects of cytokinin O-glucosylation in monocots, maize (*Zea mays*) transformants harboring the *ZOG1* gene under the control of the constitutive ubiquitin (Ubi) promoter were generated. The vegetative characteristics of hemizygous and homozygous *Ubi:ZOG1* plants resembled those of cytokinin deficiency, including shorter stature, thinner stems, narrower leaves, smaller meristems, and increased root mass and branching. On the other hand, chlorophyll levels were higher and senescence was delayed in transformants when grown in the spring/summer. Unexpected modifications in reproductive development occurred as well. Hemizygous transformants had reduced tassels with fewer spikelets but normal viable pollen. Homozygotes had very small tassels and feminized tassel florets, resembling the tasselseed

phenotype. This novel finding suggests a link between cytokinins and sex-specific floral development in monocots.

In order to better understand the dynamics of zeatin glucosylation, we studied the expression of the three major Arabidopsis glucosyltransferases, an O-glucosyltransferase (At1g22400) and two N-glucosyltransferases (At5g05860 and At5g05870) as well as three minor O-glucosyltransferases (At2g36750, At2g36790, and At2g36800) having very low specific activity with *trans*-zeatin in their native host. The spatial and temporal expression patterns in plants harboring promoter:*GUS* constructs showed some overlap, particularly in the root tips, but were also gene-specific. At1g22400 was consistently expressed in stomatal guard cells. RT-PCR indicated differential gene expression in response to stress and hormone treatments. At1g22400 was induced by drought stress supporting a possible role in stomatal control. Transformants harboring *35S:ORF:GFP* constructs showed cytosolic and nuclear fluorescence, comparable to that found for *35S:GFP* expression. Our findings demonstrate the functional significance of cytokinin glucosyltransferases by rapid regulation of active cytokinin levels in specific cell types.

Keywords: cytokinin, expression pattern, glucosyltransferase, stomata, tasselseed.
Corresponding email address: alpineda@hotmail.com

Functions and Regulation of Cytokinin Glucosyltransferases.

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Albert Pineda Rodó

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APPROVED:

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Major Professor, representing Genetics

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Director of the Genetics Program

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Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Albert Pineda Rodó, Author

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Dr. Norbert Brugière and Dr. Jeffrey E. Habben provided the back-cross seeds of the *Ubi:ZOG1* maize transformants and performed the root measurements. Maize transformation was done at Pioneer Hi-Bred International, Inc. Cytokinin analyzes were performed by Dr. Radomira Vaňková and Dr. Jiří Malbeck at the Institute of Experimental Botany v.v.i., Academy of Sciences of the Czech Republic; together with Jaleh M. Olson and Sara C. Haines from the Dept. of Horticulture at Oregon State University.

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FUNCTIONS AND REGULATION OF CYTOKININ GLUCOSYLTRANSFERASES

CHAPTER 1

INTRODUCTION TO CYTOKININS

Cytokinins are essential plant hormones for cell division and differentiation (Mok and Mok, 1994; 2001). Kinetin was the first cytokinin identified (Miller et al., 1955, 1956) as a factor stimulating cell division. Subsequently, cytokinins were found to influence a wide range of development including seed germination, *de novo* bud formation, apical dominance, leaf expansion, delay of senescence, chloroplast formation, stomatal control, reproductive development, photosynthesis, and respiration (Mok, 1994). Naturally occurring cytokinins are adenine derivatives, with isoprenoid or aromatic side chains at the N^6 position. Isoprenoid cytokinins such as *trans*-zeatin (tZ), first isolated from maize (Letham, 1963; 1964), *cis*-zeatin (cZ), N^6-(Δ^2-isopentenyl) adenine (iP), and dihydrozeatin (DZ) are major constituents in plants. In this literature review, current knowledge on cytokinin biosynthesis and metabolism will be summarized, with an emphasis on glycosylation. In addition, the influence of cytokinins on key aspects of plant development will be discussed.

Cytokinin biosynthesis

Two biosynthetic pathways of cytokinins have been reported: the *de novo* pathway and the indirect tRNA pathway (Figure 1.1). In the *de novo* pathway a key enzyme, isopentenyltransferase (IPT), catalyzes the initial and rate-limiting step in cytokinin biosynthesis. The first isopentenyltransferase was isolated from

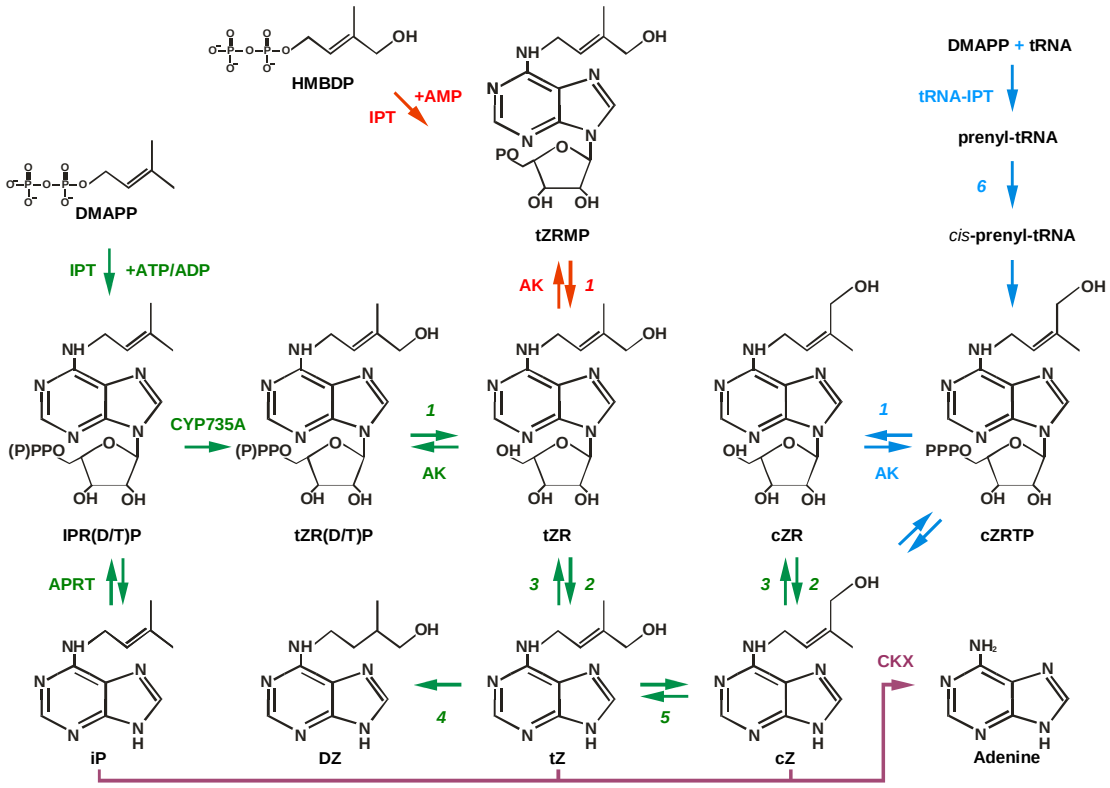

Figure 1.1 Summary of cytokinin biosynthesis pathways.

(HMBDP) 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate diphosphate; (DMAPP) dimethylallyl diphosphate; (IPT) isopentenyltransferase; (iP) N^6-(Δ^2-isopentenyl)adenine; (APRT) adenine phosphoribosyltransferase; (CYP735A) cytokinin hydroxilase; (tZR[M/D/T]P) *trans*-zeatin riboside 5'-triphosphate, diphosphate or monophosphate; (AK) adenosine kinase; (1) 5'-ribonucleotide phosphohydrolase; (tZR) *trans*-zeatin riboside; (2) adenosine nucleosidase; (3) purine nucleoside phosphorylase; (tZ) *trans*-zeatin; (4) zeatin reductase; (DZ) dihydrozeatin; (5) zeatin *cis-trans* isomerase; (cZ) *cis*-zeatin; (tRNA-IPT) tRNA-isopentenyltransferase; (6) cytokinin cis-hydroxylase; (cZRTP) *cis*-zeatin riboside 5'-triphosphate, or *cis*-zeatin nucleotide; (cZR) *cis*-zeatin riboside; (cZ) *cis*-zeatin; (CKX) cytokinin oxidase/dehydrogenase. The *Agrobacterium tumefaciens* HMBDP:AMP pathway is indicated with red arrows. The plant DMAPP:ATP/ADP pathway is indicated with green arrows. The tRNA-independent pathway is indicated with blue arrows.

the cellular slime mold *Dictyostelium discoideum* (Taya et al., 1978) and a plant isopentenyltransferase was partially purified from tobacco cell cultures (Chen and Melitz, 1979). The breakthrough, however, came with the identification of the Ti

plasmid of *Agrobacterium tumefaciens*, which was found to contain an *IPT* gene in its T-region (Akiyoshi et al., 1984; Barry et al., 1984). The IPT enzyme uses AMP and dimethylallyl diphosphate (DMAPP) to form N^6-(Δ^2-isopentenyl) AMP (IPMP). More recently a different isoprenoid donor, 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate diphosphate (HMBDP), was identified as the preferred substrate in Agrobacterium (Krall et al., 2002), which explains the high levels of tZ detected in bacteria (Akiyoshi et al., 1987). Plant *IPT* genes remained elusive for many years, but sequencing of the Arabidopsis genome led to identification of a number of genes with sequence similarity to the Agrobacterium *IPT* (Kakimoto, 2001; Takei et al., 2001b). The enzymes encoded by these plant genes, however, prefer the adenosine 5'-phosphates ADP and ATP with DMAPP as the isoprenoid precursor (Kakimoto, 2001). The tZ nucleotide form in plants is obtained through hydroxylation of the side chain catalyzed by the nucleotide-specific CYP735A hydroxylases (Takei et al., 2004a). The different nucleotides can be converted into their corresponding ribosides by 5'(3')-ribonucleotide phosphohydrolase (Polya, 1974) and into free bases by adenosine nucleosidase (Chen et al., 1981).

Based on indirect evidence, it was proposed that tZ may also be formed through an IPMP-independent pathway (Åstot et al., 2000; Rohdich et al., 2001) instead of via hydroxylation of IPMP. However, isolation of the CYP735A hydroxylases and the evidence that Arabidopsis IPTs can not efficiently utilize HMBDP as an isopentenyl precursor (Sakakibara et al., 2005) leave this pathway largely hypothetical.

The indirect biosynthetic pathway involves cytokinin production through nucleic acid breakdown, in particular tRNAs that contain cytokinins at the 3' end of the anticodon. These cytokinins include iPR, tZ riboside, cZ riboside and their methylthio derivatives, of which cZ riboside is the most abundant (Skoog and Armstrong, 1970). The first step of the indirect pathway is prenylation of tRNA catalyzed by tRNA-isopentenyltransferase (tRNA-IPT) using DMAPP as the isoprenoid precursor (Golovko et al., 2002). It is possible that this side chain is then hydroxylated by a *cis*-hydroxylase, resulting in cZ nucleotide production, completely independent from the *de novo* pathway. The rates of tRNA turn-over

only accounted up to 40% of total plant cytokinins, but recent evidence indicates that the tRNA pathway may be the main source of cZ derivatives in Arabidopsis (Kasahara et al., 2004; Miyawaki et al., 2006). However, the indirect pathway can not account for the predominance and high levels of cZ in some species or specific organs (Emery et al., 1998, Durand and Durand 1994, Veach et al., 2003). Thus the indirect tRNA pathway may be relevant to production of cZ in some plant species, but it is not a major pathway of cytokinin biosynthesis.

Plant root tips have long been considered the major sites of cytokinin biosynthesis (Letham, 1994). Cytokinins can be translocated from roots to the shoot through the xylem (Gordon et al., 1974; Duke et al., 1979; Palmer et al., 1981) and from leaves to developing inflorescences through the phloem (Taylor et al., 1990). Other actively dividing tissues, such as meristems, fruits, and seeds are also known to synthesize cytokinins (reviewed by Letham, 1994). Promoter-reporter gene studies of biosynthetic genes in Arabidopsis indicated that plant *IPT* genes are expressed in immature seeds, root tips (at early stages of emergence), and the fruit abscission zones (Miyawaki et al., 2004; Takei et al., 2004b). Expression of CYP735A hydroxylases involved in tZ biosynthesis (Takei and Sakakibara, 2005) showed a similar pattern. Studies of subcellular compartmentation of *At*IPTs revealed that the initial step of *de novo* cytokinin biosynthesis takes place in plastids (Kasahara et al., 2004; Sakakibara, 2006).

Cytokinin metabolism

Cytokinins are chemically diverse and include adenine and phenylurea derivatives (Figure 1.2). Adenine-type cytokinins with an isoprenoid side chain, including tZ, cZ, iP and DZ, are most abundant in plant tissue, but aromatic cytokinins such as N^6-benzyladenine (BAP) and its hydroxylated derivatives (topolins) have also been found in plants (Strnad, 1997). Kinetin, the first cytokinin isolated (Miller et al., 1955), is another aromatic cytokinin, containing a furfuryl ring. It is a product of autoclaving DNA (Miller et al., 1956) but may also be naturally occurring (Barciszewski et al., 1996; Barciszewski et al., 1999). The

phenylureas are synthetic cytokinins. Some phenylureas, such as N, N'-diphenylurea (DPU), are only weakly active but others, like N-(2-chloro-4-pyridyl)-N'-phenylurea (CPPU) or N-phenyl-N'-(1,2,3-thiadiazol-5-yl)urea (thidiazuron;

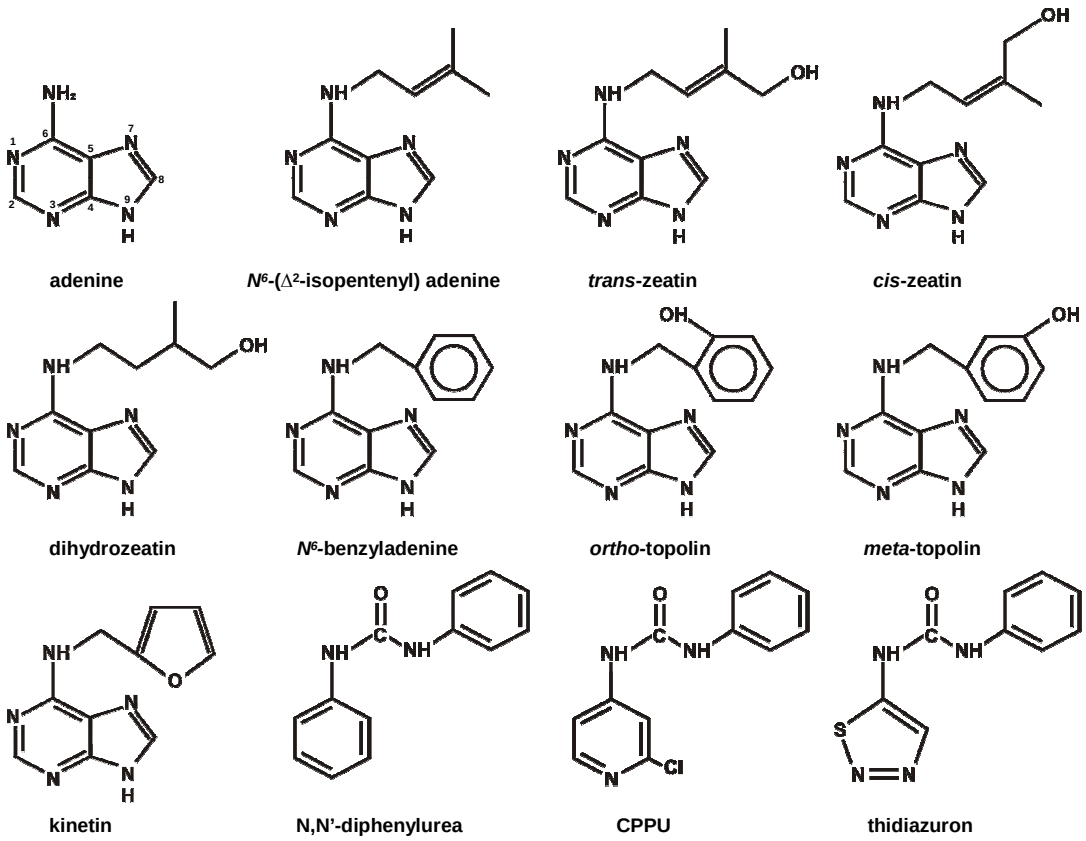

Figure 1.2 Structures of natural adenine and synthetic cytokinins.

TDZ), are highly active, even more so than purine cytokinins in certain bioassays (Mok et al., 1982).

Metabolic modifications of natural cytokinins occur on the adenine ring as well as the N^6-side chain. Changes of the adenine moiety are related to purine metabolism, mediated by enzymes with broad substrate specificity, and are likely not specific to cytokinin metabolism. Most common are conversions between free bases, ribosides, and nucleotides which primarily impact the purine salvage pathway and perhaps to a degree cytokinin homeostasis (Sakakibara, 2006).

Several enzymes have been purified and some of the corresponding genes have been isolated. They include 5'-nucleotidases, adenosine nucleosidases (Burch and Stuchbury, 1987; Chen and Kristopeit, 1981), adenosine phosphorylases, kinases and adenine phosphoribosyltransferases (APRTases) among others (Chen and Petschow, 1978: Chen et al., 1982; Moffat et al., 1992; Schnorr et al., 1996). Other conversions of the adenine ring include N-glucosylation at the *N3*, *N7* and *N9* positions (to be discussed below), and alanine conjugation at the *N9* position (Duke et al., 1978). An alanyl transferase was isolated from lupin seed which showed higher affinity to zeatin than adenine (Entsch et al., 1983).

Modifications of the N^6 side chain (Figure 1.3) are more specific for cytokinins and are relevant to biological activity. Oxidative cleavage, O-glycosylation, and reduction to dihydrozeatin are the most common. The first cytokinin oxidase (CKX) was isolated from tobacco callus tissue (Paces et al., 1971) and similar enzymes have been reported for many plant species (Armstrong, 1994; Hare and Van Staden, 1994; Jones and Schreiber, 1997; Galuszka et al., 2001). Oxidases catalyze the conversion of active cytokinins into adenine by removing their unsaturated N^6 isoprenoid side chains. Cytokinins with a saturated side chain such as DHZ are not substrates for the enzyme. Other compounds resistant to oxidase attack are cytokinin nucleotides and O-glucosides (McGaw and Horgan, 1983; Armstrong, 1994). Phenylurea cytokinins like CPPU and thidiazuron were strong inhibitors of oxidase activity. The isolation of the first maize cytokinin oxidase genes (Houba-Hérin et al., 1999; Morris et al., 1999) was followed by the discovery of homologs in Arabidopsis and *R. fascians* (Bilyeu et al., 2001). Although degradation by cytokinin oxidases was thought to require molecular oxygen, several studies have determined that the mode of action of CKXs resembles that of dehydrogenases, with cleavage under anaerobic conditions (reviewed by Galuszka et al., 2001). Therefore, CKXs are now referred to as oxidases/dehydrogenases. Promoter:*GUS* studies with the Arabidopsis *AtCKX*s (Werner et al., 2003) and the maize *Ckx1* (Brugière et al., 2003) showed

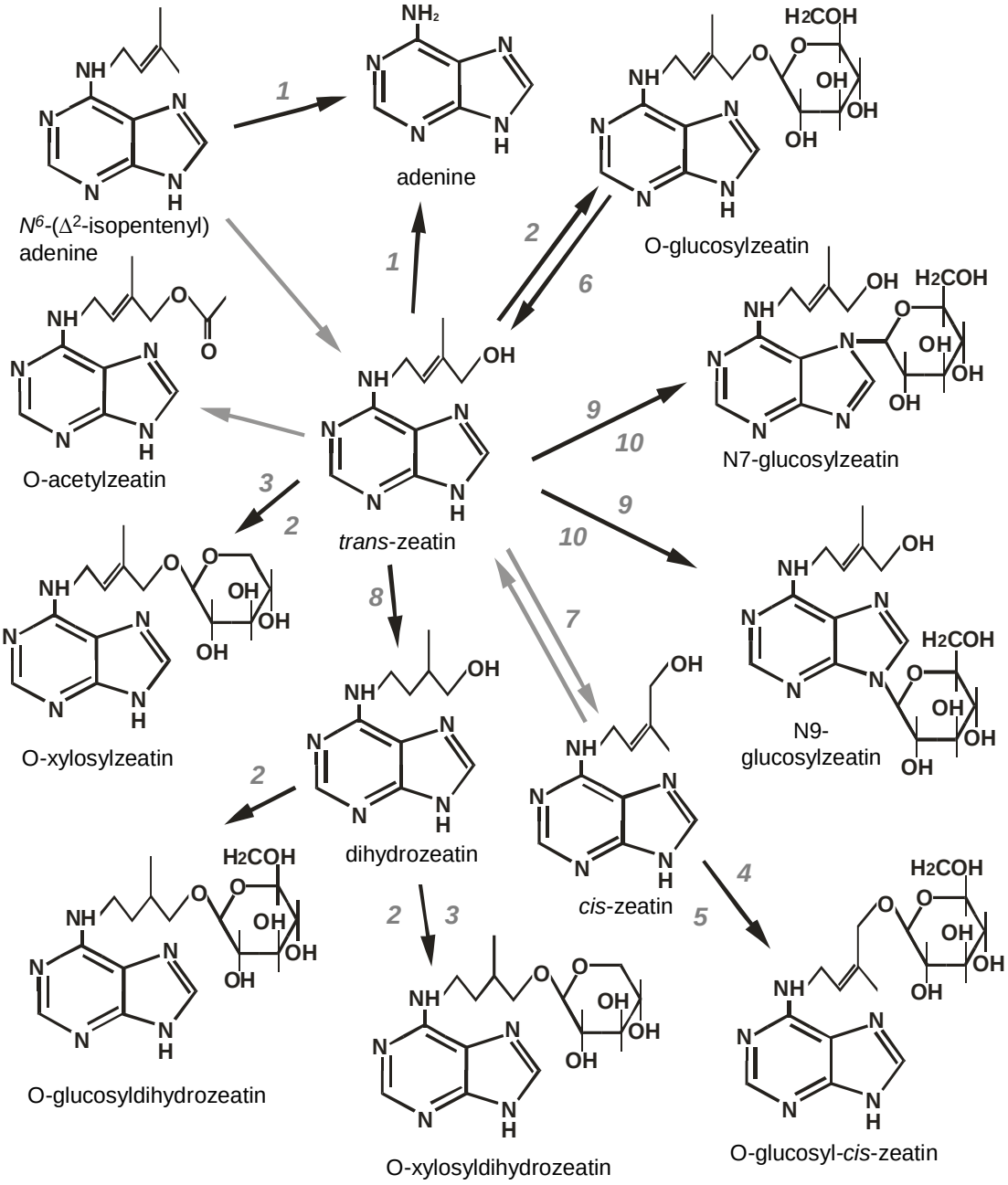

Figure 1.3 Cytokinin metabolic enzymes and genes.

(1) (*ckx*, *CKO*); cytokinin oxidase/dehydrogenase; (2) (*ZOG1*, At1g22400); *trans*-zeatin O-glucosyltransferase; (3) (*ZOX1*); *trans*-zeatin O-xylosyltransferase; (4,5) (*cisZOG1*, *cisZOG2*); *cis*-zeatin O-glucosyltransferase; (6) (*Zm-p60.1*); *β*-glucosidase; (7) zeatin *cis-trans* isomerase; (8) zeatin reductase; (9,10) (At5g05860, At5g05870); *trans*-zeatin N-glucosyltransferase. Grey arrows indicate that the conversion is known to occur, but no enzyme or gene has been isolated. No genes have been isolated for the (7,8) enzymes.

differential expression of the various genes, often including zones of active growth. Sites of expression include vascular bundles, root elongation zones, the base of reproductive organs, and even stomata. It was suggested CKXs may exert a fine tissue-specific control of catabolism which would ensure cytokinin regulation over the exit of root meristematic cells from the proliferative state (Werner et al., 2003). Subcellular localization of CKX:GFP fusion proteins revealed that most cytokinin oxidases/dehydrogenases are targeted to the secretory pathway and apoplast, supporting the hypothesis that CKXs may be secreted from the cell to control cytokinin flux through the vasculature (Galuszka et al., 2005). Others have been found in mitochondria, plastids and vacuoles (Werner et al., 2003). Intracellular CKXs were proposed to control the flux of cytokinins from biosynthetic tissue, while extracellular CKXs would perform stage-specific regulation of active cytokinins (Schmülling et al., 2003).

A reductase converting tZ to DZ was isolated from immature seeds of *P. vulgaris* (Martin et al., 1989). The enzyme did not reduce the side chains of other cytokinins such as cZ and iP. Genes encoding reductases have not been identified.

Zeatin glycosylation genes and enzymes

Glycosyltransferases (GTs) catalyze the addition of a sugar moiety to a target molecule, thereby changing its stability and solubility. Since the isolation of the first glycosyltransferase enzymes, the list of GTs has expanded to include 90 families according to current databases (http://www.cazy.org). Our study will focus on UDP-dependent glycosyltransferases (UGTs) from Family 1, characterized by their use of a uridine diphosphate (UDP) activated sugar as the sugar donor and for their C-terminal consensus sequence involved in binding the UDP moiety (Mackenzie et al., 1997; Ross et al., 2001; Shao et al., 2005). The most common sugar donor is UDP-glucose (UDPG), although other sugar donors such as UDP-galactose, UDP-rhamnose, and UDP-xylose (UDPX) are also used by certain glycosyltransferases. Acceptor substrate recognition of GTs tends to be limited to

a small range of similar molecules although some GTs are known to be more promiscuous (Entsch and Letham, 1979; Entsch et al., 1979). GTs having zeatin as substrate seem to occur universally in plants since zeatin glucosides have been found in all plants examined (Jameson, 1994; Vaňková et al., 1999).

The two main types of cytokinin glycosylations are O-linked glycosylation, where the sugar is conjugated to the oxygen of the terminal hydroxyl group of the N^6 isoprenoid side chain, and N-linked glycosylation, where the sugar moiety is attached to a nitrogen atom of the adenine ring (the *N3*, *N7* and *N9* positions). The first *trans*-zeatin O-glucosyltransferase was isolated from *Phaseolus lunatus* seed (Dixon et al., 1989) and the corresponding gene (*ZOG1*) cloned (Martin et al., 1999a). The 51 kD enzyme uses both UDPG and UDPX as the sugar donor, but has higher affinity for UDPG. A related enzyme, a zeatin O-xylosyltransferase, was obtained from *P. vulgaris* (Turner et al., 1987). This enzyme uses only UDPX as the sugar donor. The corresponding gene (*ZOX1*) was identified (Martin et al., 1999b) and shown to be 93% identical to *ZOG1*. The enzymes have high affinity for tZ (with Km of 28 μM and 2 μM for *ZOG1* and *ZOX1* respectively) but low affinity for cZ, and can also catalyze DZ to its respective O-glycosides.

Two cZ-specific O-glucosyltransferase genes, *cisZOG1* and *cisZOG2*, were obtained from maize (Martin et al., 2001b; Veach et al., 2003). The genes are 60% identical to *ZOG1* at the DNA level and the 52-kD proteins are 40% identical to *ZOG1*. The enzymes only use UDPG as sugar donor.

Additional zeatin O-glucosyltransferase genes were obtained based on screening of recombinant GT proteins from Arabidopsis for catalytic activity with zeatin (Hou et al., 2004). These genes include At1g22400, At2g36750 and At2g36800. The first gene encodes a 52-kD O-glucosyltransferase with higher activity (0.86 nkat mg^{-1}), while the other two enzymes have only low activity (0.02 and 0.03 nkat mg^{-1}, respectively) with zeatin as substrate. An additional GT, At2g36790, was found to have very low activity (0.01 nkat mg^{-1}) with zeatin (Martin RC, Mok MC and Mok DWS, unpublished results). The enzymes having low reactivity with tZ may actually be O-glucosyltransferases involved in other metabolic processes. For example, At2g36790 has high affinity to flavonoids and

is a flavonoid-7-O-glycosyltransferase (Jones et al., 2003), while At2g36800 catalyzes 23-O-glucosylation of certain brassinosteroid substrates (Poppenberger et al., 2005). At2g36750, At2g36790 and At2g36800 belong to a cluster of tandem genes with very high sequence identity, ranging from 80% to 91%.

Although cytokinin N-glycosyltransferase enzymes have been known for many years (Entsch and Letham, 1979), only recently were genes encoding such enzymes identified. In the same Arabidopsis screen that detected the O-glucosyltransferases, two N-glucosyltransferase genes, At5g5860 and At5g5870, were found (Hou et al., 2004). The genes contain ORFs of 1803bp and 1494bp and encode proteins of 50kD and 52kD respectively. The enzymes have K_ms of 220 and 240µM, and V_{max} of 4.1 and 5.9 nkat/mg for tZ, respectively. When compared to the GTs previously identified in *Phaseolus* and *Z. mays*, they show only low sequence identity (<30%). The GTs catalyze formation of both the N^7- and N^9- glucosides of a range of cytokinins (iP, tZ, DZ, BAP, and kinetin), showing much broader substrate specificity than O-glycosyltransferases.

The reverse reaction, hydrolysis of glucosides to aglycones, is catalyzed by members a different class of enzymes, the *β*-glucosidases. A maize cytokinin *β*-glucosidase was partially purified and the gene encoding it was cloned (Campos et al., 1992; Brzobohatý et al., 1993). This enzyme cleaves the sugars of zeatin O-glucoside and kinetin-N3-glucoside but not of N7- and N9-glucosides. A gene encoding a *β*-glucosidase cleaving zeatin O-glucoside was identified in *Brassica napus* (Falk and Rask, 1995).

Significance of cytokinin metabolites

Bioassays constitute the classical method for determining the activity of a compound. Free bases generally showed the highest activity in such assays (Skoog and Armstrong, 1970). However, due to rapid inter-conversions between metabolites and side-chain cleavage in plant tissues, bioassays may be misleading. Recent discoveries of cytokinin receptors (reviewed by Heyl and Schmülling, 2003 and Kakimoto, 2003) have made it possible to use alternative

methods to evaluate active cytokinins by receptor-ligand binding assays. Such studies confirmed that the free bases tZ and iP are generally the most active forms (Inoue et al., 2001; Suzuki et al., 2001; Yamada et al., 2001; Spíchal et al., 2004; Yonekura-Sakakibara et al., 2004; Maxwell and Kieber, 2004; Romanov et al., 2006). However, the different receptors vary in affinity to cytokinins. For instance, the Arabidopsis AHK3 and maize ZmHK1 receptors have high affinity for tZ, cZ and DZ whereas the Arabidopsis CRE1/AHK4 receptor prefers tZ and does not recognize cZ (Spíchal et al., 2004; Yonekura-Sakakibara et al., 2004; Mok et al., 2005). RZ is overall less active than Z and its mononucleotide again less active in the receptor assays (Spíchal et al., 2004; Yonekura-Sakakibara et al., 2004). TDZ showed very high activity in some of these assays, confirming that the adenine and urea cytokinins act through a common receptor (Shudo, 1994).

N- and O-glucosides have no cytokinin activity as determined by the receptor assays (Spíchal et al., 2004). However, while N-glucosides have no or very low activity in bioassays, zeatin O-glycosides display high activity (Letham et al., 1983a,b; Mok et al., 1992). This differential activity can be explained by the ability of *β*-glucosidases to hydrolyze O-glucosides but not N7- and N9-glucosides to their aglycones. Thus O-glucosides may be storage products to be converted back to the aglycone when cytokinin levels are too low. Furthermore, O-glucosides are found in xylem sap and may serve a role in cytokinin transport (Letham, 1994; Takei et al., 2001a).

Cytokinins and plant development

Cytokinins regulate a wide range of plant developmental processes, from seed germination to senescence (Mok, 1994). Classical studies focused on the effects of exogenous cytokinin application on plant development or on correlations between endogenous cytokinins and developmental events. With the isolation of genes involved in cytokinin biosynthesis, metabolism, and perception, forward and reverse genetic approaches have become available to more precisely dissect the functions of cytokinins. In this section, important effects of cytokinin on plant

development will be summarized. Special attention will be directed at developmental processes most relevant to Chapters 2 and 3.

De novo bud formation and release of apical dominance

Early studies by Skoog and Miller showed the marked effects of kinetin on bud differentiation in tobacco callus (Skoog et al., 1957). The relative concentrations of cytokinin and auxin were established as a strong determinant of shoot and root organogenesis in callus culture: high cytokinin/auxin ratios favored shoot formation while low cytokinin/auxin ratios favored root growth. Since then, numerous studies have confirmed that cytokinins are essential for *de novo* bud formation from callus and cultured plant tissues (e.g., Helgeson, 1968; Flick et al., 1983, Kuiper et al., 1988; Coenen and Lomax, 1997; Swarup et al., 2002).

While auxin induces apical dominance (Thimann and Skoog, 1934), exogenous and endogenous cytokinin can overcome this inhibitory effect of apical buds. A series of experiments involving decapitation and hormone applications established the interaction between cytokinins and auxins controlling bud break and lateral branching (Wickson and Thimann, 1958; Tamas, 1987; Cline, 1991). Lateral bud growth through application of cytokinins results in highly branched phenotypes, which is the principle underlying micropropagation.

Manipulations of endogenous cytokinins through genetic changes have supported these findings. Transgenic plants overexpressing the *IPT* genes displayed bushy phenotypes expected of cytokinin overproduction. These include *Nicotiana* plants harboring the Agrobacterium *IPT* gene (Smigocki and Owens, 1988; Schmülling et al., 1989; Ainley et al., 1993), Arabidopsis with their native genes *AtIPT4* and *AtIPT8* (Kakimoto, 2001; Sun et al., 2003; Miyawaki et al., 2004), and *Petunia hybrida* transformants with the Agrobacterium *IPT* (Dervinis, 1999; Clark et al., 2004).

Cytokinin deficiency was studied via insertional mutations of *AtIPT* genes (Miyawaki et al., 2006) and receptor histidine kinases genes (Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006) or by overexpressing cytokinin

oxidase/dehydrogenase genes (Werner et al., 2003; Kopečný et al., 2006). The resultant plants showed a general growth inhibition of the aerial parts, including reduced meristematic activity, thinner stems and smaller leaves as well as increased root growth.

Seed dormancy and germination

Embryonic axes of developing seeds are sites of cytokinin biosynthesis (Gepstein and Ilan, 1979; Ilan and Gepstein, 1981). Cytokinins are then transported from the embryonic axis to the cotyledons during germination (Gepstein and Ilan, 1980; Muñoz et al., 1990), which led to the suggestion that they may stimulate distribution of storage reserves to the growing seedling. Cytokinin levels in dormant seeds are low but increase during germination after a transitory decrease (Webb et al., 1979; Martin et al., 1987). This increase may be due both to cytokinin biosynthesis in the embryonic axes and possible release of active cytokinins from conjugated forms. For example, endosperm of *Phaseolus* contains very high levels of the *ZOG1*/*ZOX1* enzymes (Martin et al., 1999a; 1999b) and it is possible that the stored glycosides are hydrolyzed during germination. This is supported by a recent study with tobacco transformants harboring the promoter of the maize *β*-glucosidase gene (*ZmGLU1*) linked to the *GUS* gene, which showed that expression was low in mature seeds but increased during germination, peaking during radicle and cotyledon emergence (Gu et al., 2006). This increase in *β*-glucosidase expression suggests cytokinin reserves (O-glycosides) can be used during germination.

Leaf expansion

The stimulatory effects of cytokinins on leaf expansion were first observed with radish leaf discs (Kuraishi and Okumura, 1956). This effect on leaf expansion may be linked to the ability of cytokinins to enhance sink strength for nutrients (Gersani and Kende, 1982). Dicot transformants and mutants with decreased

cytokinin content through overexpression of cytokinin oxidase genes or decreased sensitivity by knocking out phosphorelay genes generally have smaller leaves (Werner et al., 2001; Galuszka et al., 2004; Nishimura et al., 2004; Miyawaki et al., 2006; Riefler et al., 2006). Analyses of monocots have been limited to one study of maize harboring a cytokinin oxidase under the control of a tapetum promoter in which some plants had narrower leaves (Huang et al., 2003).

Delay of senescence

A role of cytokinins in delaying leaf senescence was demonstrated in a number of studies involving exogenous cytokinin treatments (Richmond and Lang, 1957; Mothes and Engelbrecht, 1961; Thimann, 1980; Singh et al., 1992). Cytokinins were found to stimulate photosynthesis, chloroplast formation, and plastid replication (Van Staden et al., 1988; Zavaleta-Mancera et al., 1999). Furthermore, increased endogenous cytokinin levels in transgenic plants overexpressing cytokinin biosynthesis genes and mutants of CKX genes delayed senescence, increased chlorophyll retention and decreased senescence-related membrane degradation (Smart et al., 1991; Li et al., 1992; Gan and Amasino, 1995; McKenzie et al., 1998; Riou-Khamlichi et al., 1999; Ori et al., 1999; Robson et al., 2004; Clark et al., 2004; Kim et al., 2006). Contrary to expectations, transformants overexpressing cytokinin oxidase (*AtCKX2*) also had higher chlorophyll concentrations and delayed senescence (Mýtnová et al., 2006). These seemingly contradictory findings suggest that the level of cytokinins may not be the only determining factor in the senescence process.

High levels of cytokinin O-glucosides occurred in senescing tissues, suggesting that inactivation of cytokinins via glycosylation may facilitate the senescence process (Letham et al., 1983b; Taverner et al., 1999). However, there is little information on the effects of increased or decreased zeatin O-glucosylation on the senescence process. Overexpression of the maize cytokinin *β*-glucosidase Zm-p60.1 did not lead to any change in the onset of senescence (Kiran et al.,

2006), but it did not change other developmental traits either and had marginal effects on endogenous cytokinins.

Stomatal control

Cytokinins increase transpiration and stomatal aperture as demonstrated by application of cytokinins to leaves and epidermal peels (Livne and Vaadia, 1965; Incoll and Jewer, 1987; Naqvi, 1994, 1995; Pospíšilová et al., 2000) and by characterizations of transgenic plants overproducing cytokinins (Pospíšilová et al., 2000). Abscisic acid (ABA) has the opposite effect, stimulating stomatal closure (Schroeder et al., 2001). While many studies have focused on the mode of action of ABA in stomatal closing (Schroeder et al., 2001), very little is known about the mechanism of cytokinin effects on guard cells.

Long-distance signaling from roots to shoots is important in regulating responses to stress. Xylem exudates of water-stressed plants have highly increased ABA levels and lower cytokinin levels (Pospíšilová et al., 2000; Schroeder et al., 2001). Besides its important regulatory functions by itself, ABA can also induce expression of CKXs (Brugière et al., 2003), thereby decreasing cytokinin levels further.

A clear connection has been established between levels of ABA glucosides and water stress. Like ABA, ABA-glucose (ABA-GE) functions as a root-to-shoot stress signal, controlled by ABA-specific UGTs (Sauter and Hartung, 2000; Sauter et al., 2002). Stress-induced ABA-GE is first transported through the xylem to the shoots, where it can be hydrolyzed in the leaf apoplast by *β*-glucosidases (Lee et al., 2006). The genes encoding ABA-specific UGTs and *β*-glucosidases were found to be regulated by ABA and/or water stress (Xu et al., 2002; Lee et al., 2006).

Cytokinin O-glucosides may also play a role in stress signaling. Zeatin O-glucoside was found in xylem sap (Letham, 1994), and xylem sap of water-stressed tomato contained only one-half the O-glucoside levels of non-stressed controls (Davies et al., 2005). Furthermore, application of cytokinin O-glucosides

via the xylem resulted in significant increases of transpiration in derooted oat and wheat (Badenoch-Jones et al., 1996), showing that glucosides derived from the xylem sap can contribute to the active cytokinin pool in the leaves. Cytokinin-specific *β*-glucosidases have been identified, such as those in maize and *Brassica napus* (Brzobohatý et al., 1993; Falk and Rask, 1995), but involvement of the maize *β*-glucosidase in hydrolysis of cytokinin glucosides from the xylem sap may be minimal since this enzyme is located in plastids (Kristoffersen et al., 2000).

Reproductive development

The transition from the vegetative to the reproductive phase is induced by environmental factors like daylength, temperature, and phytohormones like gibberellins (reviewed by Mok, 1994). Even though there is no general agreement on the relationship between cytokinins and floral induction, cytokinins have been shown to stimulate reproductive organogenesis under the right conditions (Zeevaart, 1978). In tobacco, a shift from vegetative to reproductive growth is characterized by a general decrease in cytokinin bases and ribosides (Dewitte et al., 1999).

Evidence that cytokinins can alter sex determination in unisexual flowers has also been presented: maize ears cultured in the presence of kinetin grew male florets (Bommineni et al., 1987). However, in many other studies, cytokinins were a feminizing factor (Durand and Durand, 1994). In detailed studies with *Mercurialis*, which has genetically determined sex expression, the presence of specific cytokinins was correlated with the sex of flowers and male sterility. Only female apices had tZ, male apices had higher levels of tZR, and male-sterilility was associated with cZ accumulation (Durand and Durand, 1994).

Data from genetically modified plants complement early studies, but results are still sketchy. Cytokinin-deficient maize transformants expressing the *CKX1* gene (in the tapetum tissue) developed smaller tassels (Huang et al., 2003), while rice mutants with increased cytokinins grew more florets per panicle (Ashikari et

al., 2005), suggesting that cytokinins have a positive effect on terminal flower development. Senescence-induced expression of cytokinin in SAG-*IPT* maize lines reversed programmed abortion of the lower of the two florets, thus producing fused kernels (Young et al., 2004).

Objectives of this study

The importance of cytokinin of plant development is well documented but the role of zeatin glucosylation is unclear. The objectives of this research were:

1) To determine the effects of increased zeatin O-glucosylation on plant (maize) development;

2) To determine the expression patterns and regulation of native O- and N-glucosyltransferase genes (Arabidopsis).

Maize (*Zea mays*) transformants harboring the *ZOG1* gene (encoding a zeatin O-glucosyltransferase from *Phaseolus lunatus*) under the control of the constitutive ubiquitin (Ubi) promoter were generated to determine the morphological effects of increased glucosylation in a non-native monocot host. The central question is whether glycosylation leads to phenotypes indicative of lower cytokinin levels or that retrieval of stored O-glucosides can also result in an increase of cytokinins.

Arabidopsis thaliana plants expressing promoter:*GUS* or *35S:ORF:GFP* fusions for six cytokinin glucosyltransferases were generated in order to analyze the expression of each gene in their native host. The promoter:*GUS* fusions served to show glycosyltransferase expression in different tissues, at different stages of development, and under different conditions. The *35S:ORF:GFP* fusions made it possible to study subcellular localization of glycosyltransferase expression *in vivo.* Spatial and temporal expression patterns and factors regulating them could reveal new functions of these genes.

CHAPTER 2

OVEREXPRESSION OF A ZEATIN O-GLUCOSYLATION GENE IN MAIZE

Abstract

To study the effects of cytokinin O-glucosylation in monocots, maize (*Zea mays*) transformants harboring the *ZOG1* gene (encoding a zeatin O-glucosyltransferase from *Phaseolus lunatus)* under the control of the constitutive ubiquitin (Ubi) promoter were generated. The roots and leaves of the transformants had highly increased levels of zeatin-O-glucoside. The vegetative characteristics of hemizygous and homozygous *Ubi:ZOG1* plants resembled those of cytokinin deficiency, including shorter stature, thinner stems, narrower leaves, smaller meristems, and increased root mass and branching. Transformant leaves had higher chlorophyll content than non-transformed sibs and also higher levels of active cytokinins. Senescence was delayed when plants were grown in the spring/summer. Modifications of the reproductive phase were unexpected. Hemizygous transformants had reduced tassels with fewer spikelets but normal viable pollen. Homozygotes had very small tassels and feminized tassel florets, resembling tasselseed phenotypes. This novel finding demonstrates a link between cytokinins and sex-specific floral development in monocots.

Introduction

Cytokinins are plant hormones essential for cell division and plant development (Mok and Mok, 1994). Natural cytokinins are adenine derivatives, generally with an isoprenoid side chain at the N^6 position although cytokinins with aromatic side chains are also known to occur (Strnad, 1997). Zeatin is the central component due to its ubiquitous presence and high activity. Other free bases with cytokinin activity, *cis*-zeatin, dihydrozeatin and N^6-(Δ^2-isopentenyl)adenine, are

also present in most plant tissues. Derivatives of these bases include the corresponding ribosides and nucleotides as well as glucosides with the sugar moiety at the O of the side chain or at the *N7*, *N9*, or *N3* of the adenine ring.

Until recently, the true activities of the various cytokinin metabolites were difficult to assess since natural as well as synthetic compounds have traditionally been determined by bioassays such as the tobacco callus, bean callus, or radish cotyledon bioassays (Murashige and Skoog, 1963; Letham, 1971; Mok et al., 1978). Such tests showed the very high cytokinin activities of free bases and ribosides. However, apparent cytokinin activities may not necessarily reflect their true activities *in planta* due to rapid metabolism or catabolism. For instance, while the N7 and N9 glucosides of zeatin are mostly inactive in bioassays (Letham et al., 1983), the O-glucoside displays very high cytokinin activity, sometimes higher than zeatin itself (Mok et al., 1992). The high activity of O-glucosylzeatin was assumed to be due to conversion to the aglycone since the side chain far exceeds the optimal molecule size for cytokinin activity.

The identification of cytokinin receptors provided an alternative evaluation of cytokinin activity. For example, studies with the CRE1/AHK4 and AHK3 receptors of Arabidopsis showed that indeed, zeatin O-glucoside is not an active cytokinin (Spíchal et al., 2004). In general, these studies indicate that the highest cytokinin activity is conferred by free bases, somewhat lower activity by ribosides, and no activity by glucosides (Spíchal et al., 2004; Yonekura-Sakakibara, 2004). There are, however, differences between receptors in cytokinin recognition as illustrated by the binding of only the *trans* isomer of zeatin by the Arabidopsis CRE1/AHK4 receptor but both the *cis* and *trans* isomers by the maize ZmHK1 receptor (Yonekura-Sakakibara et al., 2004; Mok et al., 2005).

Cytokinin O-glucosides are generally assumed to be storage products. They lack activity *per se* but can be activated by *β*-glucosidase hydrolysis. Although conversion of zeatin to its O-glucoside by O-glucosyltransferases temporarily inactivates zeatin, the process also protects zeatin from cytokinin oxidases/dehydrogenases, which can degrade zeatin but not its O-glucoside (McGaw and Horgan, 1983; Armstrong, 1994). Complicating this picture is the

distribution of these metabolites in the cell, resulting possibly in differential localization of substrates and enzymes. For instance, dihydrozeatin O-glucoside and the glucosides of a number of other plant growth regulators have been found in the vacuoles (Garcia-Martinez et al., 1981; Schmitt and Sandermann, 1982; Bray and Zeevaart, 1985; Lehmann and Glund, 1986; Fußeder and Ziegler, 1988; Dean et al., 2003) whereas the maize *β*-glucosidase is targeted to plastids (Kristoffersen et al., 2000). Thus while the high activity of O-glucosylzeatin in bioassays indicates that it can be converted to zeatin, it is unclear to what extent this actually happens in plants. It is possible that conversion to zeatin is limited to certain tissues or particular stages of plant development or that it only occurs under specific conditions.

Enzymes and genes involved in zeatin glycosylation as well as glucoside hydrolysis have been identified. The first zeatin O-glucosyltransferase (EC 2.4.1.203) was isolated from immature *P. lunatus* seeds (Dixon et al., 1989) while a variant of this enzyme, a zeatin O-xylosyltransferase (EC 2.4.2.40), was obtained from those of *P. vulgaris* (Turner et al., 1987). Subsequently, the genes encoding these enzymes, *ZOG1* and *ZOX1*, were cloned (Martin et al., 1999a,b). In addition, two genes encoding O-glucosyltransferases with preference for *cis*-zeatin were isolated from maize (Martin et al., 2001b; Veach et al., 2003). Other zeatin O-glucosyltransferase genes as well as two cytokinin N-glucosyltransferase (EC2.4.1.118) genes were identified in the Arabidopsis genome (Hou et al., 2004). The enzymes all belong to Family 1 of the UDP-sugar requiring glycosyltransferases (http://www.cazy.org) and contain the signature sequence for this family in the C-proximal portion.

An enzyme converting zeatin O-glucoside to zeatin was first identified in maize (Brzobohatý et al, 1993). This *β*-glucosidase (EC3.2.1.21) has somewhat broader substrate specificity than the O-glucosyltransferases since it cleaves kinetin-N3-glucoside and some other substrates as well. The corresponding gene was cloned (*Zm-p60.1*) and found to be highly expressed in root meristems (Brzobohatý et al., 1993). A similar gene was isolated from *Brassica napus* (Falk and Rask, 1995).

We are interested in the effects of overexpressing as well as repressing *ZOG*-type genes to determine the regulatory properties of O-glucosylation on plant development. Here we report the developmental modifications of maize transformants harvoring *ZOG1* driven by the constitutive ubiquitin (Ubi) promoter. Our data indicate that zeatin O-glucosylation clearly affects root formation, leaf development, chlorophyll content, senescence, and in particular differentiation of the male flower. Generally, the transgenic phenotypes are in agreement with the hypothesis that O-glucosylation results in cytokinin deficiency. Most interesting are the effects of the *ZOG1* transgene on tassel development, with formation of tasselseed in the homozygous transformants.

Materials and Methods

Generation and selection of transgenic lines

Corn transgenic plants were obtained using an Agrobacterium-mediated transformation procedure of GS3xHC69 hybrid embryos as described previously (Zhao et al., 1998) and a construct containing the Ubi (ubiquitin) promoter and the *ZOG1* gene (accession no. AF101972). T_0 plants were backcrossed four times to HC69.

Five replications of four BC_4 lines (P1, P2, P6, and P8) were planted between the beginning of February and April and grown in the greenhouse at $25^oC/20^oC$ (day/night) under natural light. Plants were identified as bialaphos-resistant or –sensitive by painting part of the lower leaf with 200 mg l^{-1} bialaphos. For each replication, data were obtained from four plants per BC_4 line.

Crosses between sibs of the BC_4 lines produced progeny equivalent to F_2s. These progenies segregated at the expected 3:1 ratio of bialaphos resistant to bialaphos sensitive plants. Three repeats of two F_2 lines (P3 and P7) with 30 plants per line were planted in early October to study the F_2 generation. The photoperiod was gradually extended by using high-pressure sodium lamps (Hortilux LU1000B/Ht1/En) to simulate day length conditions in spring and

summer (April planting). Two additional 30-plant replications of P3 and P7 were planted in the spring and grown under natural light.

Distribution of Materials

Novel materials described in this publication may be available for non-commercial research purposes upon acceptance and signing of a material transfer agreement. In some cases such materials may contain or be derived from materials obtained from a third party. In such cases, distribution of material will be subject to the requisite permission from any third-party owners, licensors or controllers of all or parts of the material. Obtaining any permission will be the sole responsibility of the requestor. Plant germplasm and transgenic material will not be made available except at the discretion of the owner and then only in accordance with all applicable governmental regulations.

PCR

DNA was isolated from 100-mg tissue samples by sequential treatment with 500µl extraction buffer (200mM Tris-HCl, 250mM NaCl, 25mM EDTA, 0.5% SDS), 500µl saturated phenol, and 500µl chloroform, followed by precipitation with 400µl isopropanol. *ZOG1* internal primers ZOG411F (5'-CATCTCAAATGTTGAA-AACTAC-3') and ZOG930B (5'-CTTCACTTCCGGCAAAGATGTC-3') were used for PCR. After an initial denaturation at 95℃ for 4 min, 30 cycles of: 95℃ for 1 min, 52℃ for 1 min, and 72℃ for 1 min were run. An additional cycle at 72° C for 10 min was run and then the sample was kept at 4^{o}C.

RT-PCR

Total RNA was isolated from leaves, roots, and tassels at various stages using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) according to the

manufacturer's instructions. Polysaccharide-rich samples were treated with 20% PVP and 8M lithium chloride and incubated overnight at -20℃. Synthesis of cDNA was achieved using the SuperScript^{TM} II reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA). Resulting cDNA product was subjected to semi-quantitative PCR using actin to normalize samples. Primers for actin PCR were mzACTIN-32F (5'- GTGACAATGGCACTGGAATG-3') and mzACTIN-741B (5'-GACCTGACCATCAGGCATCTC-3'). The internal primers ZOG411F and ZOG930B were used to amplify *ZOG1*. The following conditions were used for PCR: initial denaturation at 94℃ for 4 min followe d by 30 cycles of 94℃ for 1 min, 57℃ for 45 sec, and 72℃ for 1 min. The last cycle was followed by incubation at 72℃ for 10 min.

Western analyses

Protein was extracted from leaves with buffer containing 55mM Tris, 50 µM EDTA, 5 mM DTT, pH 7.4 (800µl extraction buffer for 200mg leaf tissue) followed by 30%-75% NH_4SO_4 fractionation. Samples were desalted and then purified by Blue Sepharose 6B affinity chromatography (Dixon et al., 1989). A fraction equivalent to 7 mg fresh leaf tissue was separated on a 12% SDS polyacrylamide gel and blotted to Immobilon-P transfer membrane (Millipore, Billerica, MA, USA). Western blots were developed as described in Martin et al. (1990).

Phenotypic characterizations

Phenotypic characterization of transformed lines included measurements on vegetative growth rate, plant height, root length, chlorophyll content, leaf width, stomatal distribution, meristem structure, development of reproductive organs (time of appearance, size), pollen viability, and kernel development.

Foliar data presented were taken from the 10th leaf, which was found to be representative of the whole plant by measuring all leaves from fewer plants (data not shown). Foliar chlorophyll levels were monitored with a chlorophyll content

meter CCM-200 (Optisciences Inc., Tyngsboro, MA, USA) over a 175-day period. Four measurement points were taken from each leaf. A standard curve was made to convert these measurements to chlorophyll units per leaf fresh weight. Chlorophyll was extracted by incubating leaf tissues in N,N'-dimethylformamide (7% leaf weight/ solvent volume) at 4℃ for 48h, as described in Inskeep and Bloom (1985). Sample absorbances were measured at 647nm and 664.5nm (maximum for chlorophyll b and a respectively) and absolute chlorophyll contents calculated according to the equation: total chlorophyll (mg l^{-1}) = $17.90*A_{647}$ + $8.08*A_{664.5}$. Optimeter CCI units were then transformed into µg of chlorophyll per mg fresh weight with restriction curves. Random leaf samples were examined with a light microscope to determine stomatal and pavement cell distribution patterns.

Apical meristem samples were collected from 30-day-old plants, fixed in FAA (50% ethanol, 10% acetic acid, 5% formalin), dehydrated in a graded ethanol series, and embedded in Technovit 7100 plastic (Heraeus Kulzer, Wehrheim, Germany). Sections were cut on an AO 820 rotary microtome to obtain 46-µm-thick serial sections. Samples were stained with Toluidine Blue-O, and analyzed with Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA).

Pollen viability was tested prior to pollination assays. Fresh pollen grains were collected at 9 am and kept for 2h on liquid germination media containing (l^{-1}) 236.2 mg $Ca(NO_3)_2$, 24.8 mg H_3BO_3 and 30% (w/v) of PEG. Silk filaments from the female flower were added to enhance pollen tube growth. A pollen cryo-preservation protocol was optimized to ensure a constant supply of viable pollen during pollination assays. Pollen grains were collected, dried at 20℃ and 20-40% humidity for 2 h and shock-frozen by immersion in liquid nitrogen as described by Barnabas (1994). A saturated MgCl solution was used to stabilize the humidity in the desiccator. Prior to use, pollen was thawed in a water bath at 40℃ for 2 min. The combination of fresh and cryopreserved pollen allowed uniform pollination coverage during the receptive period of the maize ear.

The experimental design for all phenotypic traits was fully randomized. Data were analyzed statistically by multivariate analysis of variance (MANOVA) with repeat, line, and type as the main sources of variation, followed by a Tukey

test at 5% significance level. The analyses aimed to identify the interactions among the independent variables as well as their effect on the response variables. For those traits where no relevant variable interactions were observed, two-way and one-way ANOVA was performed.

Cytokinin analyses

Cytokinin levels were determined in leaves and xylem sap of the non-transformed, hemizygous, and homozygous plants. Leaves were taken from 95-day-old plants in late July. Xylem sap was collected from 110-day old non-transformed and hemizygous plants. For extraction and purification of cytokinins as well as determination of cytokinin levels the procedures described by Veach et al. (2003) were followed. The HPLC/MS system consisted of a HTS-Pal auto-sampler with a cooled sample stack (CTC Analytics, Zwingen, Switzerland), quaternary HPLC pump Rheos 2200 (Flux Instruments, Basel, Switzerland), Delta Chrom CTC 100 Column oven (Watrex, Prague, Czech Republic) and TSQ Quantum Ultra AM triple-quad high resolution mass spectrometer (Thermo Electron, San Jose, USA). For HPLC a Synergi Hydro column (Phenomenex, Torrance, USA) was used. Data were obtained from three samples per genotype, with two HPLC injections and analyses per sample.

Results

Transgenic *Ubi*:*ZOG1* maize lines were generated using Agrobacterium-mediated transformation. Bialaphos-resistance was the selectable marker and initial transformants were backcrossed to parental inbred for four generations to generate transformants with genetic backgrounds similar to the parental inbred. Four BC_4 lines (P1, P3, P6 and P7), derived from independent transformation events and containing a single insert each, were selected for further study. The BC_4 lines segregate into bialaphos-resistant hemizygous (P+) and bialaphos-sensitive non-transformed (NT) plants. To obtain homozygous transformants

(P++), hemizygous BC_4 plants were selfed. For simplicity, the progeny obtained after selfing the hemizygous BC_4 is referred to as F_2.

Bialaphos-resistant plants transcribe and translate ZOG1

RT-PCR (Figure 2.1a) and Western (Figure 2.1b) blots indicated that transcription and translation of *Ubi:ZOG1* occurs in transgenic maize tissues. Tissue samples from bialaphos-resistant plants of all four lines produced clear RT-PCR and protein bands but those from bialaphos-sensitive plants did not.

Increased cytokinin conjugation leads to pronounced changes in maize plant architecture and leaf width

The hemizygous BC_4 plants were morphologically distinguishable from non-transformed sibs. They grew slower, were shorter, and had narrower leaves. There were three types of F_2 plants, bialaphos-sensitive non-transformed plants, plants resembling the hemizygous BC_4 transformants, and a class of extremely small plants (Figure 2.2a). The segregation ratios were 16:36:10 and 15:27:22 in the P3 and P7 lines respectively, fitting a 1:2:1 ratio and thus supporting the hypothesis that the very small plants were homozygous for the transgene. There were significantly fewer leaves on the hemizygous transformants than in the non-transformed plants and even fewer leaves on the homozygous transformants (Figure 2.2b). At the time of tassel emergence, plant height was reduced by 22% to 41% in the hemizygous and by 48% to 60% in the homozygous *Ubi:ZOG1* plants compared with non-transformed plants (Figure 2.2c). There was a greater reduction in plant height than in the number of leaves, indicating that internodes were shortened in response to transgene activity. Stems were slender in hemizygous and very slender in homozygous Ubi:*ZOG1* plants. The leaves of hemizygous plants were significantly narrower than those of non-transformed sibs (Figures 2.2d,e), but their length was the same (data not shown). The leaves of homozygous transformants were extremely narrow, even at plant maturity (Figure

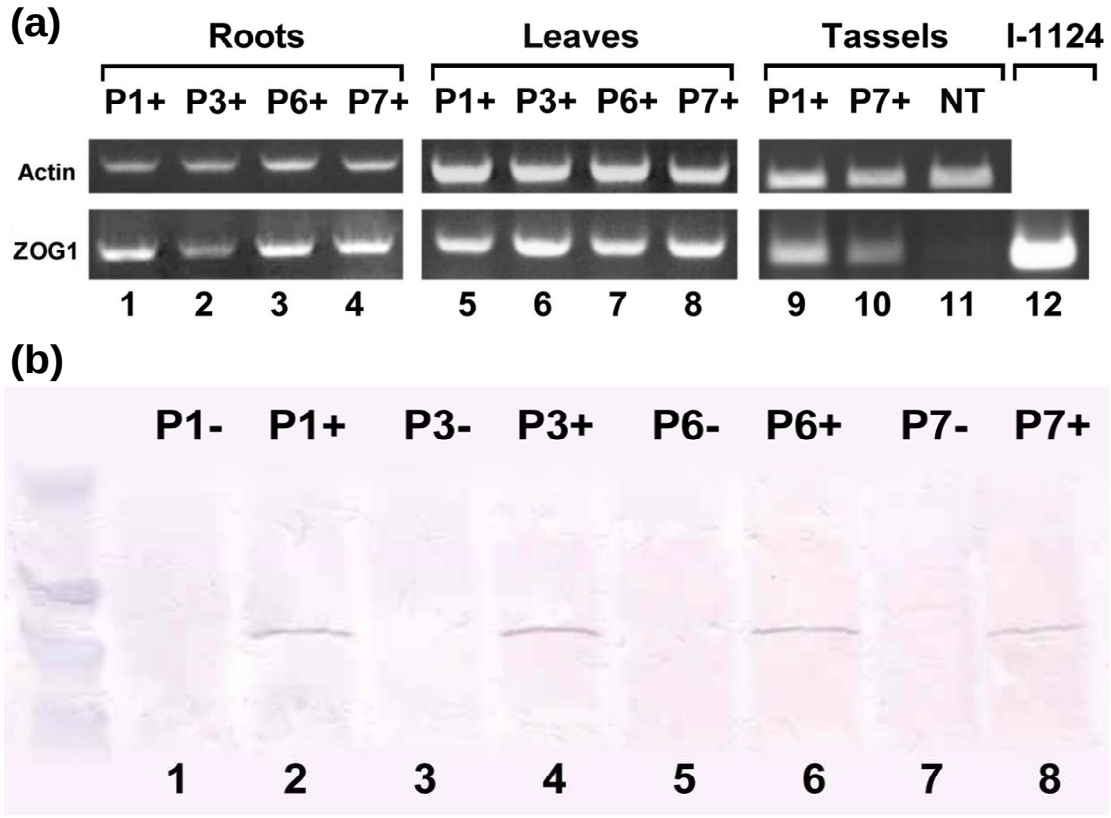

Figure 2.1 *Ubi:ZOG1* expression.

(a) RT-PCR products with *ZOG1*-specific primers and actin as a control showing expression of the transgene in roots, leaves, and tassels. P1+, P3+, P6+ and P7+ are independent hemizygous lines. NT is a non-transformed control. Plasmid I-1124 contains the *Ubi:ZOG1* insert. (b) Western blot of ZOG1 product in leaves (4 mg equivalent) from hemizygous transformants and non-transformed sibs, developed with monoclonal antibodies specific to ZOG1.

2.2e), and also shorter. The thinner stems and leaves of transformants suggested smaller meristematic regions and microtome sections confirmed that hemizygous and homozygous meristems were indeed significantly smaller (Figures 2.2f,i). None of these characteristics were influenced by environmental fluctuations (light intensity or day length).

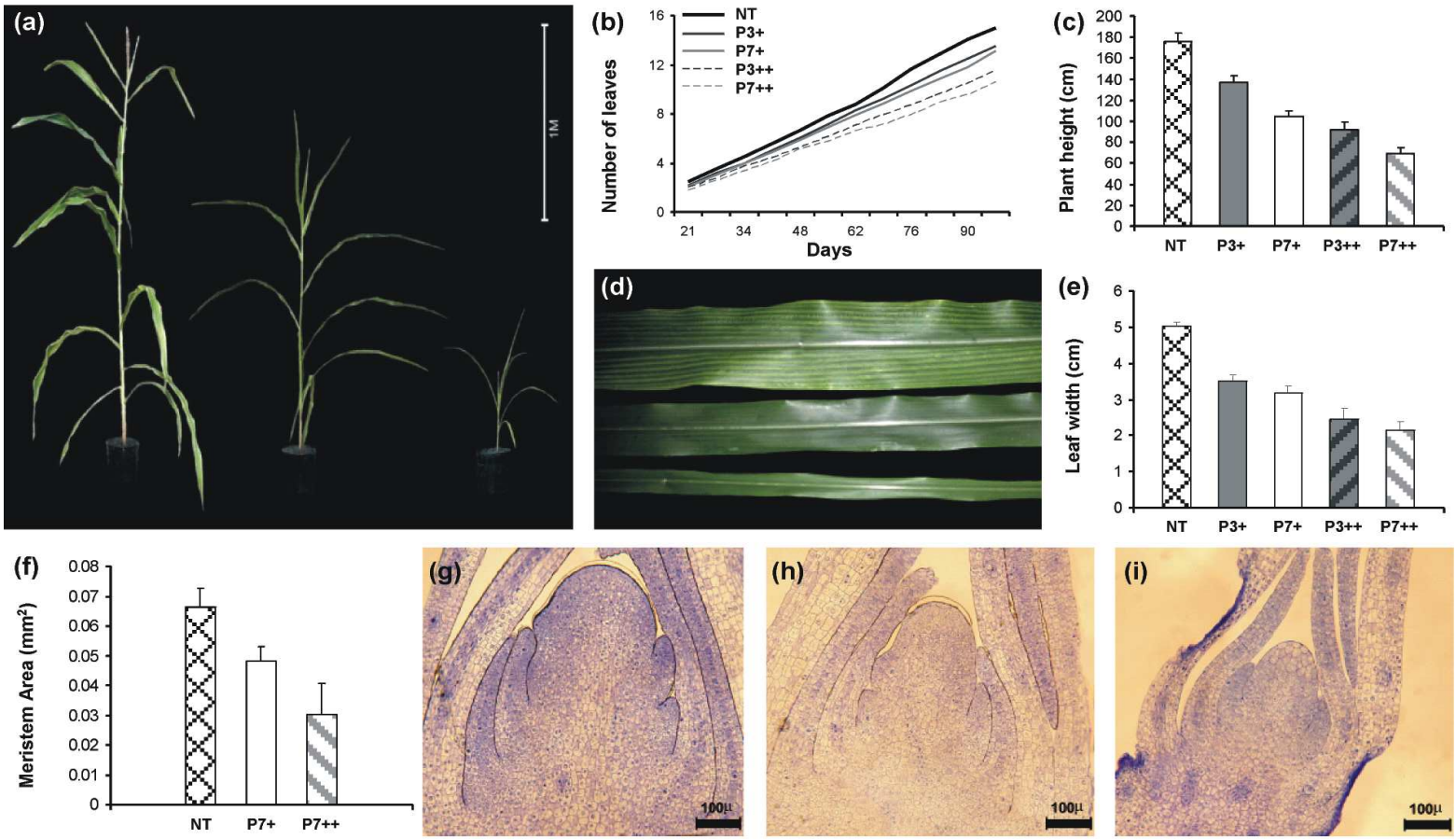

Figure 2.2 Vegetative development. (a) Non-transformed (left), hemizygous (center) and homozygous (right) 97-day-old P7 plants. (b) Number of leaves over time. P+ and P++ represent hemizygous and homozygous lines respectively. (c) Plant height at tassel emergence. (d) Non-transformed (top), hemizygous (center) and homozygous (bottom) P7 leaves. (e) Average width of the tenth leaf. (f) Shoot meristem size of 40-day-old plants. (g) Non-transformed meristem. (h) P7 Hemizygous meristem. (i) P7 Homozygous meristem.

Chlorophyll formation and distribution of stomates are altered in transformants

Differences in foliar chlorophyll were noticeable as early as 23 days after germination. The transformed plants were darker green than the non-transformed ones. Chlorophyll levels in transformants were elevated throughout their life span and ultimately resulted in the delay of senescence that kept transformed plants green weeks after control sibs had died (Figure 2.3a). This trait was influenced by the environment since delayed senescence was observed in the summer but not in the winter under artificial light (Figure 2.3b), even though the day length was adjusted to mimic summer conditions. It is most likely that the difference in light intensity and/or quality was a major factor.

Closer examination of the leaves revealed that the number of major veins was the same in non-transformed plants and hemizygous transformants but that the distance between the veins was smaller in the latter. On normal maize leaves, the stomata usually occur in rows, both in double files with stomata at alternate positions (Figure 2.3c; Hernandez et al., 1999) and single files (Figure 2.3d). Transformants had fewer double file stomata (Figure 2.3e) but more single files. The overall density of the stomata in the leaves did not differ between the two groups (Figure 2.3f).

Root mass and branching is increased in Ubi:ZOG1 transformants

Root morphology differed between the WT and hemizygous transformants. *Ubi:ZOG1* roots were thicker, more branched (Figure 2.4a) and longer (Figure 2.4b). As a result, the total root weight was much greater in the transformants (Figure 2.4c).

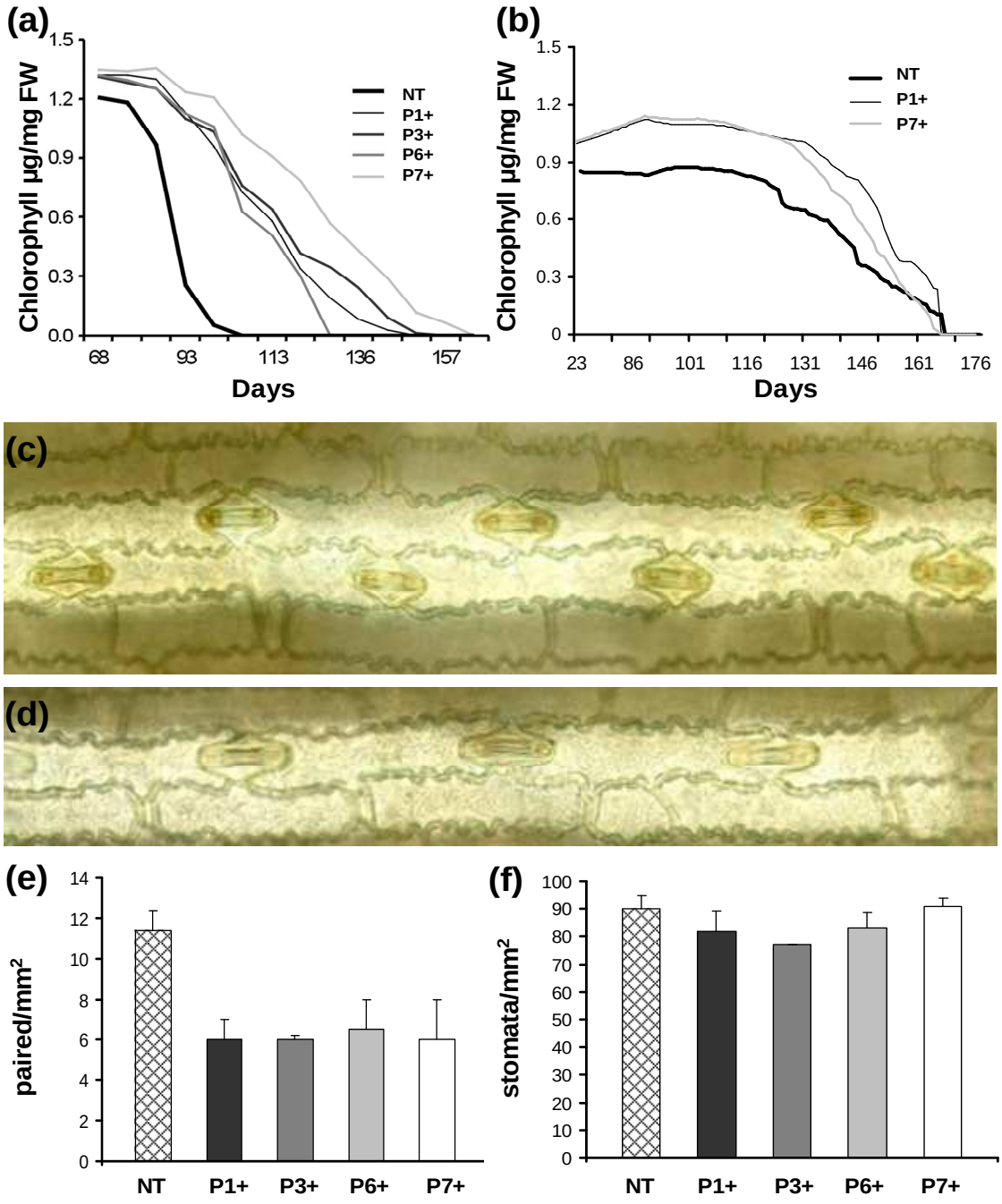

Figure 2.3 Leaf characteristics.

(a,b) Chlorophyll levels in the 10th leaf through senescence. Plants grown in the spring/summer under natural long-day conditions (a) and in the fall under artificial lighting (b) (c) Paired chain of stomata from a non-transformed leaf. (d) Single chain of stomata from *Ubi:ZOG1* leaf. (e) Number of paired stomata per mm^2 (f) Total number of stomata per mm^2.

Ubi:ZOG1 causes reduction of tassel size and feminization of florets

Hemizygous *Ubi:ZOG1* plants showed delayed tassel initiation (Figure 2.5a) and more strikingly, a drastic reduction in tassel size, branching, and spikelet production (Figure 2.5b,d). The weight of transformed tassels was about 75% less than that of non-transformed tassels (Figure 2.5b), due to the decreased number and smaller size of branches. Moreover, the tassels were abnormal. While normal maize tassels have many florets up to the tip of each branch (Figure 2.5e), the hemizygous *Ubi:ZOG1* tassels had functional spikelets only at the lower end of the tassels while the tips of the branches were devoid of most floral structures (Figure 2.5f). Sterile spikelets consisted of two external glumes with no florets, lemna or stamens. The normal florets underwent anthesis and produced functional pollen grains as demonstrated by the ability of the pollen to germinate (Figure 2.5g). Pollen viability was further supported by the roughly equal numbers of transformed and untransformed kernels in the BC_4 populations, which were generated using BC_3 transformants as male parents.

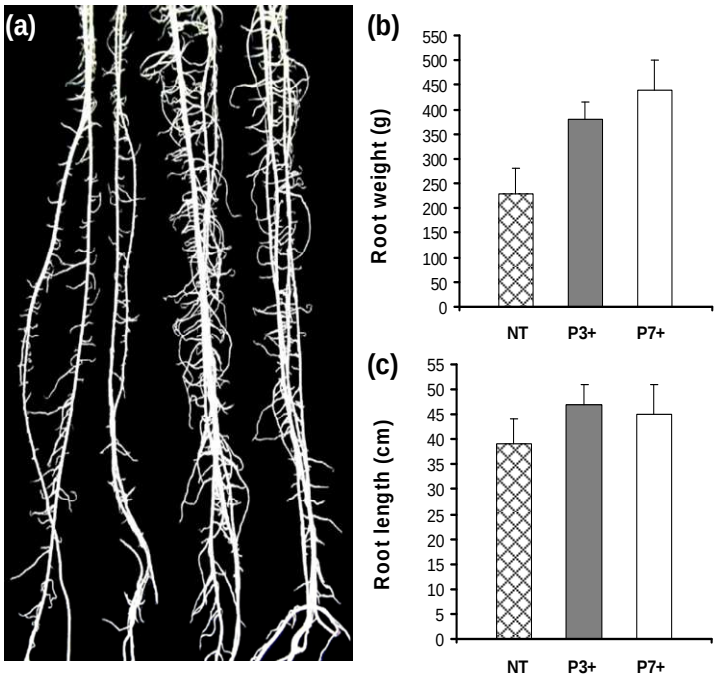

Figure 2.4 Root development.

(a) Non transformed (two at left) and hemizygous (two at right) roots 3 weeks after planting. (b) Root fresh weight. (c) Root length.

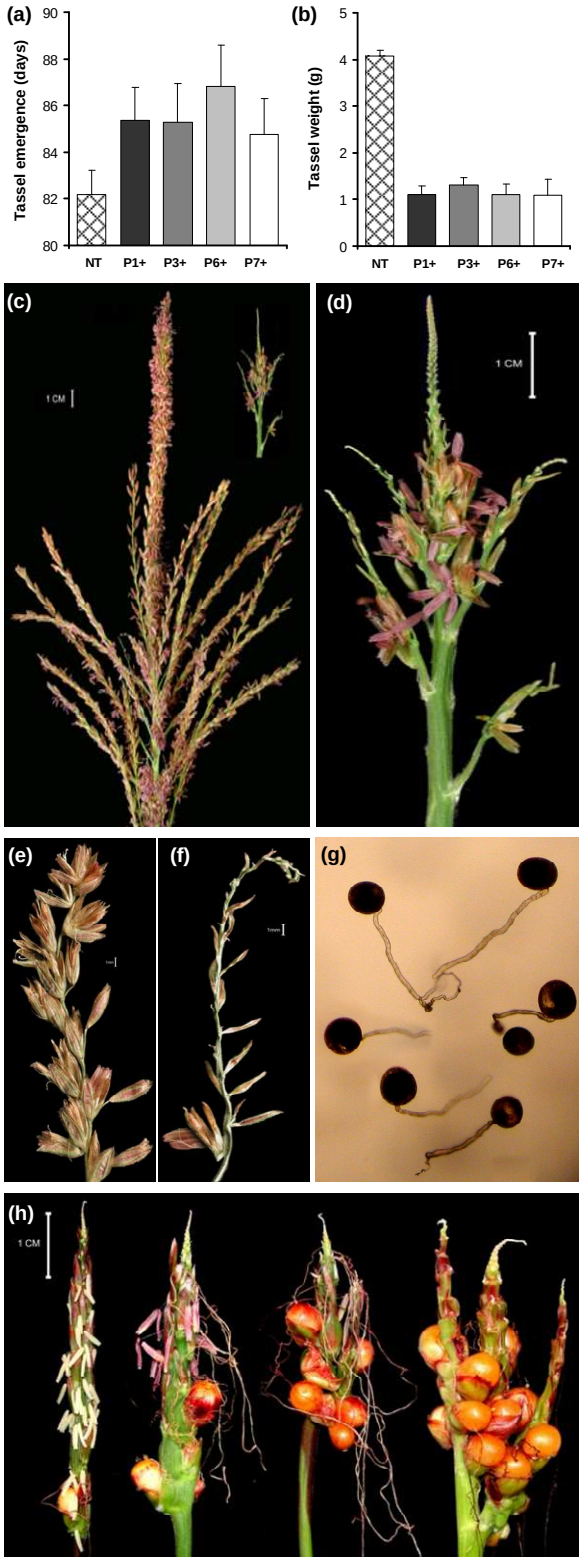

Figure 2.5 Tassel development.

(a) Age of plant at tassel emergence (b) Tassel fresh weight at maturity (c) Non-transformed tassel (d) Hemizygous *Ubi:ZOG1* tassel. (e) Control lateral rachis with fully developed fertile spikelets. (f) Hemizygous lateral rachis with empty spikelets. (g) Viable *Ubi:ZOG1* pollen in liquid germination medium. (h) Homozygous tassels with various degrees of floret feminization.

Homozygous F_2 tassels were phenotypically even more extreme, showing a dosage effect of the *ZOG1* gene. They were very small, had no or very few branches, and most interestingly, showed various degrees of floret feminization (Figure 2.5h). Fertilization of these female florets resulted in formation of complete kernels (Figure 2.5h). The feminization varied in the homozygous population, from a few female florets and seeds to almost complete silking of the tassel (Figure 2.5h, left to right). Normal tassel floret development is initially bisexual, after which florets become unisexual through gynecium abortion (Cheng and Pareddy, 1994). This programmed gynoecium

abortion was inhibited by ZOG1 overexpression in some of the lower florets since fertile ovules and elongated silk were formed. The few male florets on the apical portion of the homozygous tassels yielded a small amount of viable pollen. The phenotype was dependent on the light condition. All plants grown in the winter under artificial light always showed some degree of feminization while plants grown in the spring and summer under ambient light conditions had only a few tasselseeds.

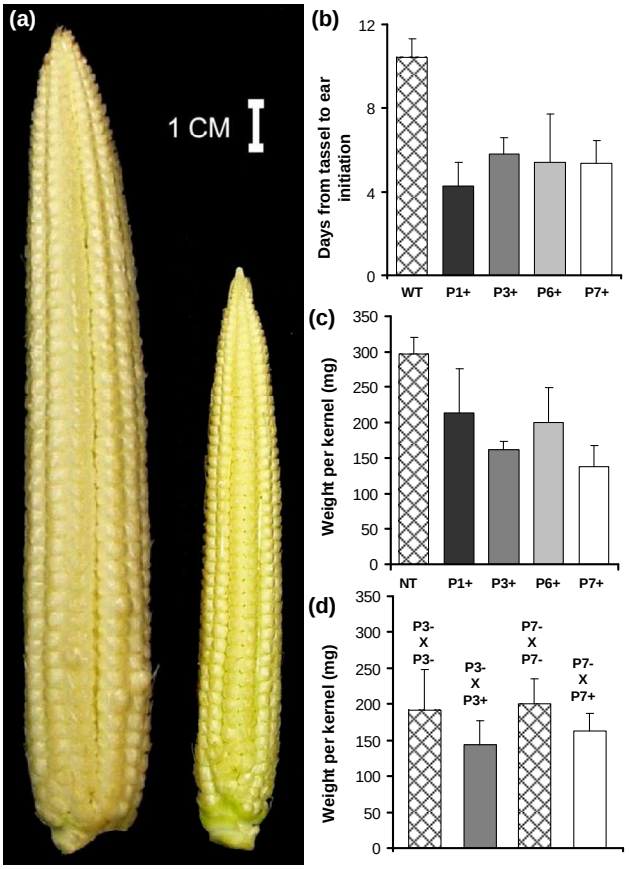

Figure 2.6 Ear and kernel development.

(a) Non transformed (left) and hemizygous (right) ears of the same age (b) Days from tassel to ear initiation. (c) Weight of kernels from non-transformed ears pollinated with pollen from non-transformed (NT) or hemizygous *Ubi:ZOG1* (P+) plants. (d) Weight of kernels from non-transformed ears pollinated with pollen from hemizygous transformants. Kernels were taken from the same ear and classified as P- X P- or P- X P+ by PCR with *ZOG*1-specific primers.

Ubi:ZOG1 reduces seed weight

Ear development on hemizygous *Ubi:ZOG1* plants lagged behind that of non-transformed plants but the ears looked normal (Figure 2.6a). The time between tassel and ear emergence was slightly longer in non-transformants than transformants (Figure 2.6b). Hemizygous ears had only partial seed set, in contrast to fully filled non-transformed ears under the same greenhouse conditions; however, when the ears were artificially pollinated, the hemizygous

ears were also completely filled, indicating that pollen availability (not viability) was the limiting factor. When the *Ubi:ZOG1* plants were used as the male parent, plants produced kernels with a lower average weight than those pollinated with non-transformed pollen (Figure 2.6c). To confirm this initial observation, hemizygous plants were used to pollinate non-transformed plants and the resulting kernels were weighed and classified as transgene positive or negative (using PCR with *ZOG1*-specific primers). The data indicate that kernels on the same ear resulting from pollination with *Ubi:ZOG1* pollen grains were significantly smaller than those resulting from pollination with non-transformed pollen (Figure 2.6d).

Cytokinin concentrations in Ubi:ZOG1 plants and non-transformed sibs

Cytokinins were analyzed in roots and leaves of 28-day-old and 95-day-old plants (Figure 2.7). Roots of young plants had very low levels of free bases and there were no significant differences between transformed and non-transformed plants in the levels of any of the compounds (Figure 2.7a). The levels of all root ribosides together was slightly lower in the transformants than in non-transformed plants (Figure 2.7b). In leaves of both young and older plants, the levels of free bases were significantly higher in transformants than non-transformed sibs (Figures 2.7d,g). Also ribosides were increased by the presence of the transgene, but more in younger than older plants (Figures 2.7e,h). As in leaves, the levels of both free bases and ribosides were higher in xylem sap of transformants (Figure 2.7j,k). Since the levels of dihydrozeatin and its derivatives were generally below detection levels, they were not reported. The level of cytokinin nucleotides was significantly higher in leaves and xylem sap of older transgenic plants than in non-transformed plants (Figure 2.7h,k) but the levels were not significantly different in roots and leaves of young plants (Figure 2.7b,e). All together, the data indicate that the levels of active cytokinins (free bases and ribosides) are about the same

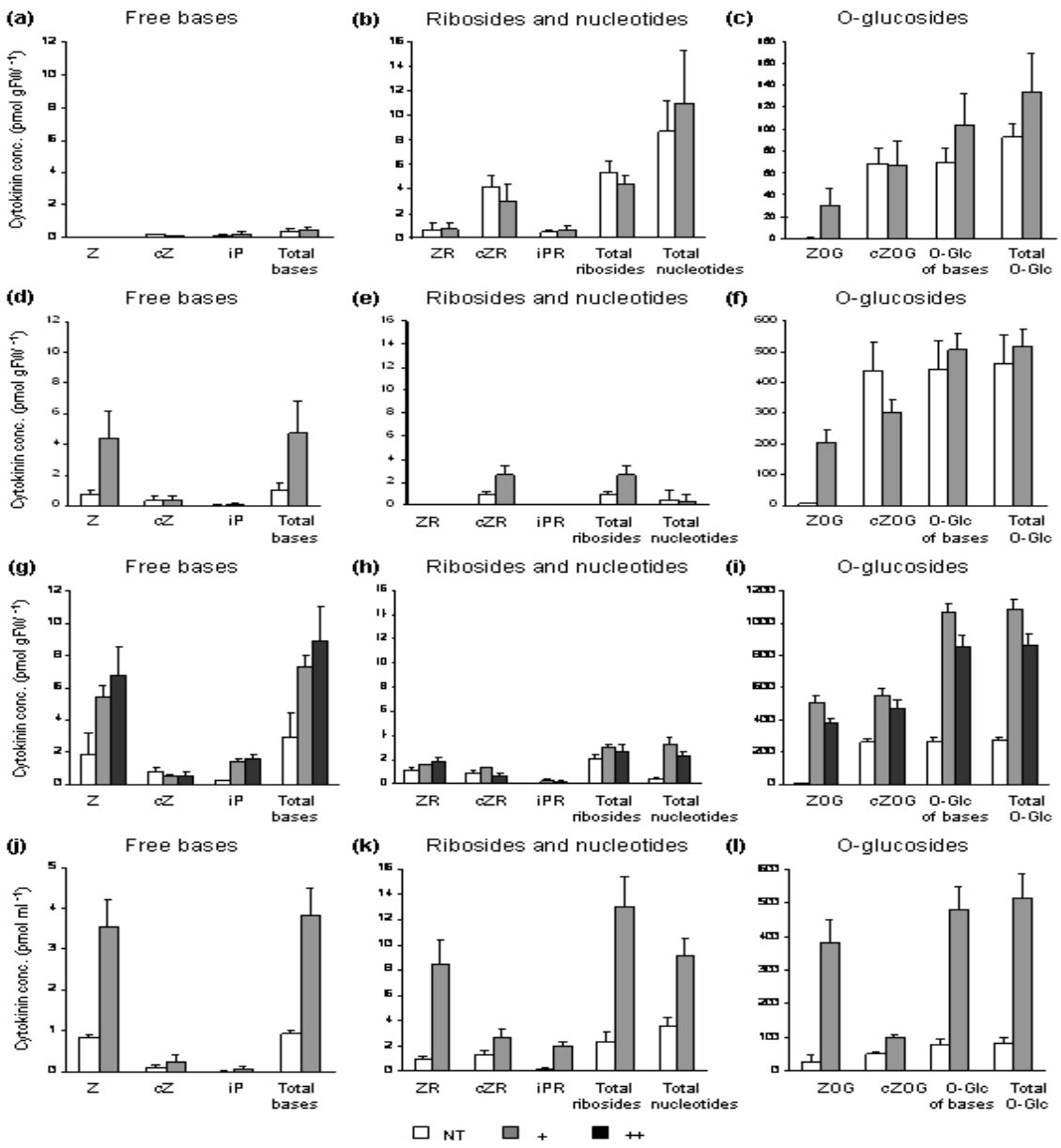

Figure 2.7 Cytokinin concentrations in leaves and xylem sap.

(a,b,c) Cytokinin concentrations in roots of 28-day-old non-transformed (NT), hemizygous *Ubi:ZOG1* (+) and homozygous *Ubi:ZOG1* (++) maize plants. (d,e,f) Cytokinin concentrations in leaves of 28-day-old maize plants. (g,h,i) Cytokinin concentrations in leaves of 95-day-old maize plants. (j,k,l) Cytokinin concentrations in xylem sap of 110-day-old maize plants. (a,d,g,j) Free bases; (b,e,h,k) ribosides and nucleotides; (c,f,i,l) O-glucosides. Z, *trans*-zeatin; *c*Z, *cis*-zeatin; iP, N^6-(Δ^2-ispentenyl)adenine; ZR, *trans*-zeatin riboside; *c*ZR, *cis*-zeatin riboside; iPR, N^6-(Δ^2-ispentenyl)adenosine; ZOG, *trans*-zeatin O-glucoside; *c*ZOG, *cis*-zeatin O-glucoside.

or slightly lower in roots but higher in leaves of transformed plants than non-transformed plants. It should be noted that at the time the xylem sap was collected, the transformants were physiologically younger than the non-transformed plants and thus the higher levels of active cytokinins in transformants could reflect this physiological difference.

O-Glucosides were by far the most abundant class of cytokinin metabolites in all tissues, even in non-transformed plants where the O-glucoside of *cis*-zeatin was the main component. As expected, the level of zeatin O-glucoside was increased significantly in the *ZOG1* transformants: 100-fold higher in roots, 38 and 88-fold higher in leaves of young and older plants, respectively, and 15-fold higher in xylem sap of hemizygous plant than non-transformed plants. In contrast, the O-glucoside of *cis*-zeatin was the same in roots and leaves of young hemizygous *Ubi:ZOG1* plants and controls, and only about two-fold higher in leaves and xylem sap of the older plants. This is expected since conversion rates of *cis*-zeatin by *ZOG1* are extremely low (Mok et al., 2005). Levels of free base O-glucosides far exceeded those of ribosides even though ribosides were generally more abundant than free bases. This is in agreement with the substrate preference of the ZOG1 enzyme for the free base, but it also indicates that ribosylation of O-glucosides is sporadic. The levels of N-glucosides were negligible compared to those of O-glucosides (data not shown).

Discussion

Overexpression of the *ZOG1* gene in maize led to a number of marked developmental changes such as decreased stature, thinner stems, narrower leaves, increased root branching and higher chlorophyll. The most interesting effect of the *ZOG1* gene was the change in reproductive development, with integration of female and male florets in the terminal inflorescence in the homozygous F_2 plants. The results will be discussed in relation to the known effects of cytokinins on plant development and the literature on mutants and overexpressers involving cytokinin-related genes.

Early vegetative development of the transformants reflects lower levels of active cytokinins

The hemizygous *Ubi:ZOG1* transformants were shorter and less robust than the controls and this reduction was more severe in the homozygotes, indicating dosage effects of the transgene. The slower growth and reduced plant size are consistent with reports of dicots having lower cytokinin levels or sensitivities due to increased expression of oxidases/dehydrogenases (Werner et al., 2003; Kopécný et al., 2006), deficiencies in biosynthesis (Miyawaki et al., 2006), or mutations in receptor genes (Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006).

Observations regarding cytokinin effects on monocot leaf development are limited. Classical experiments involving exogenous cytokinin applications conducted with dicots demonstrated that cytokinins stimulated leaf expansion (Kuraishi and Okumura, 1956). Transformants and mutants of Arabidopsis with either reduced cytokinin levels or deficient cytokinin perception have smaller leaves in many cases, but the ratio between the length and width remained constant (Riefler et al., 2006; Werner et al., 2001; Werner et al., 2003). In maize, the width of the leaves of hemizygous plants was significantly reduced by overexpression of *ZOG1* while in homozygous plants both width and length decreased. Maize genetic mutants with smaller leaves usually have smaller meristems (Scanlon et al., 1996). This is also the case in the *ZOG1* transformants. The meristems of the hemizygous plants were slightly smaller than those of the controls and the homozygous meristems were even smaller. Similarly, the meristems were also smaller in Arabidopsis plants having lower cytokinin (Werner et al., 2003; Kopečný et al., 2006). Another notable difference between the *ZOG1* transformants and control plants was the arrangement of leaf stomata. There were more single files and fewer double files in the transformants, which may be related to the smaller number of cell files between veins.

While shoot development was inhibited by the presence of the transgene, root development was enhanced in the *Ubi:ZOG1* maize plants, similar to Arabidopsis and *Lotus japonicus* transformed with maize cytokinin oxidase/dehydrogenase genes (Werner et al., 2001, 2003; Lohar et al., 2004; Kopečný et al, 2006). The active cytokinin levels were lower in roots of the *Ubi:ZOG1* transformants than controls. This suggests that the unavailability of physiologically active cytokinins caused by increased glucosylation stimulated the initiation and growth of root tips.

Later vegetative development of the transformants reflects higher levels of active cytokinins

The higher chlorophyll and delayed senescence in transformant leaves are indicative of an increase in active cytokinin since exogenous cytokinins and upregulation of cytokinin biosynthesis produce similar phenotypic changes (Richmond and Lang, 1957; Smart et al., 1991; Li et al., 1992; Gan and Amasino, 1995; Robson et al., 2004). The cytokinin analyses confirm that active cytokinins (free bases and ribosides) are increased in leaves. It is possible that accumulation of high amounts of O-glucosides can eventually lead to increased free bases due to the action of *β*-glucosidases, thereby causing the delay in leaf senescence.

There is a similar observation of higher chlorophyll and delayed senescence in a recent report of tobacco overexpressing the *AtCKX2* gene of Arabidopsis (Mýtnová et al., 2006). The tobacco transformants contained higher xanthophyll levels which may have provided greater photoprotection. A factor common to both the transgenic maize and tobacco is the generally slower development due to cytokinin O-glucosylation and degradation respectively, prolonging the growth period. However, if this slower development is delaying maturation to a significant extent, also time of flowering would be greatly delayed. This was not the case since appearance of tassels was only about 2 days later in transformants than untransformed controls. Leaf senescence showed a much longer delay (Figure 2.3a). Both the *ZOG1* and *AtCKX2* transformants may

respond to the increased conjugation of the aglycone by increasing cytokinin biosynthesis. Since biosynthesis takes place in the plastids (Kasahara et al., 2004; Sakakibara, 2006), there may be a localized increase of cytokinins in plastids, causing the elevated chlorophyll levels.

It can not be excluded that the higher chlorophyll in the maize leaves are related to indirect effects. Non-transformed maize leaves expand very rapidly under natural summer conditions while those of the transformants remain much narrower, which could result in differential chlorophyll accumulation. Furthermore, the ratio between root and shoot growth is much higher in the transformants, possibly leading to higher accumulation of nutrients (including N) in the leaves. If this is the case, the higher cytokinins in the mature leaves could merely be a consequence of the delayed senescence.

Increased cytokinin conjugation leads to defective tassel development and feminization of the lower florets

The drastic reduction in tassel size of the *Ubi:ZOG1* transformants points to the importance of cytokinins in maize reproductive development. Normal tassels have 10 to 50 lateral branches which contain paired spikelets (Cheng and Pereddy, 1994). In contrast, the hemizygous *Ubi:ZOG1* transformants had fewer and shorter lateral branches. Terminal florets were often missing. Also maize transformed with the *CKX1* gene driven by a pollen-specific promoter had smaller tassels even though *CKX1* expression was targeted to the pollen (Huang et al., 2003). A rice cultivar with reduced expression of a cytokinin oxidase/dehydrogenase gene and increased cytokinins had more reproductive organs (Ashikari et al., 2005). Although reproductive programs of maize and rice are different in a number of aspects, these studies suggest that cytokinins have a positive effect on terminal flower development, most likely through its effects on meristem size.

Most intriguing was the occurrence of female florets at the lower end of the homozygous *Ubi:ZOG1* tassels which formed seed when pollinated. Maize florets

begin as complete bisexual flowers containing pistil and anther initials, but later the pistils abort (Cheng and Pareddy, 1994). The process of pistil abortion must have been inhibited in the homozygous *Ubi:ZOG1*. It should be noted that tasselseeds were found on all four transformed lines examined indicating that they were not a consequence of accidental insertion in one of the tasselseed (*TS*) loci but rather as the result of altered cytokinin levels or composition. Tasselseed in maize is a known phenomenon and several genetic mutations causing feminization of the tassel have been described (Irish and Nelson, 1989; Dellaporta and Calderon-Urrea, 1994). The most prominent phenotypes occur in recessive mutants ts1/ts1 and ts2 /ts2, which display complete reversion from male to female inflorescences, with failure of pistil abortion and induction of stamen abortion (Emerson, 1920; Irish et al., 1994; DeLong et al., 1993). Somewhat less extreme is the dominant Ts5 mutant, which shows positional effects, with female florets occurring at the basal portion of the tassel (Nickerson and Dale, 1955), similar to the *Ubi:ZOG1* transformants. The ts4/ts4 and ts6/ts6 mutants also show partial reversions (Dellaporta and Calderon-Urea, 1994). The *TS2* gene was cloned and found to have homology to short chain alcohol dehydrogenases (DeLong et al., 1993). The maize transformants described here establish the first link between tasselseed and cytokinins.

Previously, cytokinins have been implicated in sex expression of a number of plants (Durand and Durand, 1994) but to our knowledge have not been researched in connection to sex expression in maize tassels. The occurrence of tasselseed on the homozygous *Ubi:ZOG1* transformants indicates that increased zeatin O-glucosylation and the associated disturbance in cytokinin homeostasis result in feminization. Whether this is due to a decrease in active cytokinins (as evidenced by the retarded shoot development and smaller meristem size) is difficult to assess. It should be noted though that tasselseed formation was also observed on maize transformants overexpressing a cytokinin oxidase/dehydrogenase gene under the control of the Ubi promoter (N. Brugière, unpublished results). In most plant species (although primarily dicots), cytokinins are feminizing, but exceptions are known (Durand and Durand, 1994).

Interestingly, only the lower inflorescences showed this feminization whereas the more apical florets had the usual male characteristics. Thus there may be a gradient of active cytokinins or some polarity in signal distribution, causing female flowers to develop at the basal end of the tassel.

A remote but possible alternative explanation for the tasselseed characteristic could reside in the changed composition of cytokinins. Not much is known about the influence of particular cytokinin metabolites on sex expression. In the most extensively studied system, *Mercurialis*, where a number of genes control sex expression, specific cytokinins have been linked with sex differentiation and male sterility. For instance, occurrence of *trans*-zeatin in apices was correlated with femaleness, while its riboside and nucleotide were more abundant in males (Durand and Durand, 1994). The presence of *cis*-zeatin and its riboside were associated with male sterility. The changes in the concentrations of the various cytokinin metabolites in the transgenic maize and the changed ratios between *cis* and *trans* isomers may lend some support to this hypothesis.

The influence of light intensity or quality on the tasselseed trait in *Ubi:ZOG1* transformants indicates an interaction between the cytokinin and light signaling pathways. Such interactions have been observed previously. For instance, exogenous cytokinin caused de-etiolation of dark-grown Arabidopsis seedlings (Chory et al., 1994). Furthermore, Arabidopsis transformants and mutants with altered expression of the cytokinin type A response regulator *ARR4* showed altered red light sensitivity (Sweere et al., 2001; To et al., 2004).

Pollen grains of hemizygous *Ubi:ZOG1* plants appeared normal and germinated on artificial medium. Even the few anthers on homozygous transformants contained normal pollen. Also Arabidopsis transformants with constitutive overexpression of *CKX* genes had fewer but functional pollen (Werner et al., 2003); however, maize plants overexpressing *CKX1* via a pollen-specific promoter (pZtap) showed pollen sterility (Huang et al., 2003).

Female inflorescence development is normal but seeds are smaller in Ubi:ZOG1 plants

The female inflorescences are not altered in *Ubi:ZOG1* maize plants indicating that the changes in cytokinin levels have stronger effects on male flower development. There were also no obvious abnormalities in the flowers of transgenic tobacco overexpressing *ZOG1* (Martin et al., 2001a) even though tobacco flowers are derived from the terminal bud, nor in plants constitutively overexpressing *CKX* genes (Werner et al., 2003). However, when cytokinins levels were increased through senescence-induced expression of the Agrobacterium *IPT* gene, the pistil was retained in the lower floret and fused kernels were formed (Young et al., 2004).

Until recently, very few studies have addressed effects of cytokinin on seed size. Transgenic Arabidopsis with decreased cytokinin levels or sensitivity produced fewer but larger seeds (Werner et al., 2003; Kopečný et al., 2006; Riefler et al., 2006). In the present study, seeds with increased cytokinin conjugation were smaller than non-transformed seeds from the same ears when control plants were pollinated with pollen from hemizygous *Ubi:ZOG1* transformants. Whether this is due to lower or higher cytokinin levels is not clear. Maize and Arabidopsis seed development can not be compared due to the fact that maize kernels are largely endosperm while Arabidopsis seeds consist mainly of embryos.

Implications of endogenous cytokinin composition

As expected, the zeatin O-glucoside levels were very high in leaves of transformants. Interestingly, they were also high in the xylem sap indicating that O-glucosides are transported from the roots to the shoots, perhaps particularly when there is overproduction in the roots. This is in agreement with Letham's review of the literature pointing to O-glucosides in xylem sap along with ribosides, free bases and nucleotides (Letham, 1994). Notably, O-glucosides were also identified in xylem sap of wheat, a monocot related to maize.

In the present study, the predominant cytokinin in non-transformed maize leaves was the O-glucoside of *cis*-zeatin. In a previous study, *cis*-zeatin and its derivatives were found to be the major components in maize roots and stems (Veach et al. 2003). Levels of *cis* isomers were also very high in kernels although lower than the *trans* counterparts (Veach et al., 2003). Maize has a cytokinin glucosyltransferase with preference for *cis*-zeatin over *trans*-zeatin (Martin et al. 2001b). In addition, a maize cytokinin receptor, ZmHK1, with high affinity for *cis*-zeatin and *trans*-zeatin has been identified (Yonekura-Sakakibara et al, 2004). These findings suggest that *cis*-zeatin is an active cytokinin in tissues where ZmHK1 is expressed.

A related issue is the possible origin of *cis*-zeatin. A hydroxylase for the synthesis of *cis*-zeatin, similar to the hydroxylase found in Arabidopsis for the formation of *trans*-zeatin (Takei et al., 2004a), is a possibility. Recent findings in Arabidopsis (Miyawaki et al., 2006) favored tRNA degradation rather than de novo synthesis as the source of *cis*-zeatin. Maize may be different from Arabidopsis in that the *cis*-zeatin level is too high to be accounted for solely by tRNA breakdown. In addition, the presence in maize of a receptor responsive to *cis*-zeatin and a *cis*-specific glucosyltransferase indicates species-specific pathways, supporting that direct synthesis of *cis*-zeatin may occur in monocots.

Conclusions

The changes in plant architecture associated with *ZOG1* overexpression are consistent with reduction of active cytokinins, but later developmental changes, particularly associated with chlorophyll levels and retention, seem to reflect increases in cytokinins. This is supported by the cytokinin analyses of the plants. The modifications in the reproductive phase, specifically the feminization of tassel floret development, were unexpected. This novel observation provides a link between cytokinins and sex-specific floral development in maize.

CHAPTER 3

REGULATION OF ZEATIN GLUCOSYLTRANSFERASE GENE EXPRESSION IN ARABIDOPSIS

Abstract

Cytokinins can be conjugated to N- and O-glucosides. While N-glucosylation generally leads to irretrievable loss of activity, O-glucosides may play an important role in cytokinin storage and homeostasis since they can be hydrolyzed to the corresponding aglycones. The first zeatin O-glycosyltransferase genes isolated were *ZOG1* and *ZOX1* from beans (Martin et al., 1999a,b), and more recently cytokinin O- and N-glucosyltransferase genes were identified in Arabidopsis (Hou et al., 2004). In order to better understand the dynamics of zeatin glucosylation, we studied the expression of the three major Arabidopsis glucosyltransferases, an O-glucosyltransferase (At1g22400) and two N-glucosyltransferases (At5g05860 and At5g05870) as well as three minor O-glucosyltransferases (At2g36750, At2g36790, and At2g36800) having very low specific activity with *trans*-zeatin in their native host. The spatial and temporal expression patterns in plants harboring promoter:*GUS* constructs showed some overlap, particularly in the root tips, but were also gene-specific. At1g22400 was consistently expressed in stomatal guard cells. RT-PCR indicated differential gene expression in response to stress and hormone treatments. Most interestingly, At1g22400 was induced by drought stress. Transformants harboring *35S:ORF:GFP* constructs showed cytosolic and nuclear fluorescence, comparable to that found for *35S:GFP* expression. Our findings indicate the functional significance of cytokinin glucosyltransferases in rapidly changing active cytokinin levels in specific cell types.

Introduction

Cytokinins are plant hormones regulating cell division and plant development (Mok and Mok, 1994; 2001). They influence many aspects of plant growth, including seed germination, bud formation, apical dominance, leaf expansion, stomatal opening, chlorophyll formation, and senescence. Naturally occurring cytokinins are adenine derivatives with an isoprenoid or aromatic side chain at the N^6 position (Skoog and Armstrong, 1970; Strnad, 1997). The most common and active isoprenoid cytokinin is *trans*-zeatin (tZ), although other free bases like N^6-(Δ^2-isopentenyl) adenine (iP), *cis*-zeatin (cZ) and dihydrozeatin (DZ) are also present in most plant tissues. Modifications of the adenine ring or the N^6-side chain lead to formation of ribosides and nucleotides as well as O- and N-glucosides at the isoprenoid hydroxyl group and the *N3*, *N7* or *N9* position of the adenine ring respectively.

Cytokinin bases and ribosides have long been considered the active forms based on bioassays (Murashige and Skoog, 1962; Letham, 1971; Mok et al., 1978; Skoog and Armstrong, 1970). However, O-glucosides also have apparent biological activity (Letham et al., 1983b; Mok et al., 1992). These classical assays are indirect and may not reflect real activities due to metabolic conversions of the compounds applied. The recent identification of cytokinin receptors (Kakimoto, 2003) facilitates more direct and rapid assays based on ligand-receptor binding. Studies with Arabidopsis and maize receptors confirmed that tZ and iP have the highest biological activity, followed by the ribosides, but showed that glycosides are inactive (Yamada et al., 2001; Spíchal et al., 2004; Yonekura-Sakakibara, 2004). Therefore, the high activity of O-glucosides in bioassays must be due to conversion to the active aglycones via hydrolysis by cytokinin *β*-glucosidases (Campos et al., 1992; Brzobohatý et al., 1993; Falk and Rask, 1995). O-glucosylation confers resistance to cytokinin oxidases/dehydrogenases (CKXs), which cleave the isoprenoid side chain. Thus O-glucosides may serve a as a cytokinin storage form.

Several enzymes and genes mediating cytokinin glycosylation have been identified. A zeatin O-xylosyltransferase and a zeatin O-glucosyltransferase were isolated from *Phaseolus vulgaris* and *P. lunatus* respectively (Dixon et al., 1989;

Turner et al., 1987), and the corresponding genes, *ZOX1* and *ZOG1*, were cloned (Martin et al., 1999a,b). Two O-glucosyltransferases (*cisZOG1* and *cisZOG2*) with preference for *cis*-zeatin were identified in maize (Martin et al., 2001a; Veach et al., 2003). One major cytokinin O-glucosyltransferases (At1g22400) was identified in Arabidopsis through a screen of recombinant glucosyltransferases for activity with zeatin (Hou et al., 2004). Two additional O-glucosyltransferases, At2g36750 and At2g36800, had very low activity with zeatin (Hou et al., 2004) and another member of this gene cluster, At2g36790, had trace activity (Martin RC, Mok MC and Mok DWS, unpublished results). These weak zeatin O-glucosyltransferases have other main substrates, such as flavonoids (for At2g36790) and brassinosteroids (for At2g36800) (Jones et al., 2003; Poppenberger et al., 2005).

The first zeatin N-glucosyltransferase was isolated from radish by Entsch and Letham (1979). Screening of recombinant Arabidopsis glycosyltransferase led to identification of two zeatin N-glucosyltransferase genes (At5g05860 and At5g05870) (Hou et al., 2004). These enzymes all belong to Family 1 of the UDP-dependent glycosyltransferases (UGTs), characterized by their use of uridine diphosphate (UDP) activated sugar donors and their UDP-binding C-terminal consensus sequence (Mackenzie et al., 1997; Ross et al., 2001; Shao et al., 2005).

Transformation of several plant species with *ZOG1* was useful for identifying its effect on plant development (Martin et al., 2001b). However, studies of *ZOG1/ZOX1* expression through promoter-reporter constructs were hampered by the inability to transform beans. The recent identification of similar Arabidopsis genes offered the opportunity to use this model system for gene expression studies. In this chapter we describe the spatial and temporal expression of the six Arabidopsis zeatin glucosyltransferases using promoter:*GUS* fusions. Furthermore, regulation of the most important genes (At1g22400, At5g05860, and At5g05870) by abiotic stress and hormones was determined by RT-PCR (short-term) and GUS expression (long-term). The most significant finding is the ubiquitous expression of At1g22400, the major zeatin O-glucosyltransferase, in guard cells. Its expression was enhanced by drought, heat, and abscisic acid

(ABA). As cytokinins are known to promote stomatal aperture (Livne and Vaadia, 1965; Incoll and Jewer, 1987), this finding implicates zeatin O-glucosylation in guard cell control.

Materials and Methods

Overview of experiments

Six Arabidopsis Family 1 UGT glycosyltransferase genes (At2g36750, At2g36790, At2g36800, At1g22400, At5g05860 and At5g05870) encoding enzymes with zeatin glucosyltransferase activity were studied. For gene expression, promoter:*GUS* constructs were generated and transgenic plants obtained. Spatial and temporal gene expression was based on patterns of GUS stain. The effects of stress and hormones were determined on expression of At1g22400, At5g05860, and At5g05870 since the enzymes they encode have the highest activity. In addition, the sub-cellular localization of these gene products was determined using transgenic plants harboring *35S:ORF:GFP* constructs.

Plasmid construction and sequence verification

To prepare promoter:*GUS* constructs, genomic fragments containing the putative promoters (upstream regions up to the border of the next gene, ranging from 663bp to 1760bp) plus the 5' untranslated region (UTR) were amplified from genomic DNA of *Arabidopsis thaliana* ecotype Columbia. For the *35S:ORF:GFP* constructs, the entire open reading frames (ORFs) of At1g22400, At5g05860 and At5g05870 were amplified without the stop codon. In order for the amplicon to be suitable for Gateway recombinational cloning, all forward (F) primers contained the attB1 sequence and all reverse (R) primers the attB2. The attB flanking tails as well as the 20bp gene-specific sequences for each primer are presented in Table 3.1. *Pfu* DNA polymerase was used in PCRs according to the manufacturer's instructions (Promega Corporation, Madison, WI, USA). The

Table 3.1 Primer list for the Gateway cloning reactions.

All forward primers (F) include the attB1 sequence tail and all reverse primers (R) contain the attB2 sequence tail. (F and R) primers amplify promoters; (ORF F and R) primers amplify whole ORFs.

Name	Sequence
attB1	5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTTG-3'
attB2	5’-GGGGACCACTTTGTACAAGAAAGCTGGGTG-3’
At2g36750 F	5’-TTGTTGTTTGTTAAATCATTGTGC-3’
At2g36750 R	5’-ATGAGGAACTCTCTTCTAAGG-3’
At2g36790 F	5’-TTTGTACGAATTAGCAGAAGAC-3’
At2g36790 R	5’-GAACAAGATGCGTGGAGAG-3’
At2g36800F	5’-CCATCCAGGTTTTTGTGCTT-3’
At2g36800 R	5’-CTCAAGTTTTGCTTTGGTTTTG-3’
At1g22400 F	5’-GACGTGGCATTATTCTCGTG-3’
At1g22400 R	5’-CTTTCTCTTGCGCGAAATCAG-3’
At5g05860 F	5’-ATTGGTTCGTGTCTCATGTCC-3’
At5g05860 R	5’-ATTGTAATTGAAACGCTCTAG-3’
At5g05870 F	5’-AGTTGGGAATATTTGCTAAAG-3’
At5g05870 R	5’-TCAAGTTTCTTTGTTCTTCC-3’
At1g22400 ORF F	5’-ATGGGATCTCAGATCATTCAT-3’
At1g22400 ORF R	5’-ATCCTGTGATTTTTGTCCCAA-3’
At5g05860ORF F	5’-ATGGAGGAGAAGAGAAATGG-3’
At5g05860ORF R	5’-CAACAATAGTATATGATTAGC-3’
At5g05870ORF F	5’-ATGGAGAAGAGAAACGAGAG-3’
At5g05870ORF R	5’-CGTAGGCACTAG-TGGCTCG-3’

standard Gateway PCR consisted of an initial denaturation at 94℃ for 4 min, 30 cycles of 94℃ for 45 sec, Tm℃ for 45 sec and 72℃ for elongation. An additional cycle at 72℃ for 10 min was run and then the sample was kept at 4℃. Annealing temperatures ranged from 56 to 63℃ depending on the primer set. The elongation cycle ranged from 1 to 3.5 minutes depending on amplicon size. The amplified fragments were cloned into the Gateway pDONR207 vector and transfected into a One Shot ® Top 10 competent *E. coli* according to the instruction manual of Gateway™ Cloning Technology (Invitrogen, Carlsbad, CA, USA) and Walhout et al., (2000). Vectors were purified with a Perfectprep plasmid mini kit (Eppendorf, Westbury, NY, USA) and the sequence and orientations of the inserts were verified by PCR and DNA sequencing. A unique set of external pDONR primers was used for PCR of all donor vectors (Table 3.2). The pDONR amplification program consisted of an initial denaturation at 95℃ for 4 min, 30 cycles of 95℃ for 1 min, 48 for 1 min, and 72℃ for 1 min. An additional cycle at 72℃ for 10 min was run and then the sample was kept at 4℃. Vectors were also sequenced on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) using the same pDONR primers.

After verification of the sequences, the promoter/5'UTR fragments were recombined into pGWB3 in-frame upstream of the β-glucuronidase (GUS) gene. The ORF fragments were recombined into pGWB5 in-frame between the 35S promoter and sGFP, a codon-optimized green fluorescent protein with enhanced spectral qualities (Chiu et al., 1996). The pGWB binary vectors are based on Clontech Gateway technology, developed and kindly provided by Dr. Tsuyoshi Nakagawa of Shimane University.

Transformation of Arabidopsis

The vectors were introduced into the EHA105 strain of *Agrobacterium tumefaciens* (Hood et al., 1993). The sequences of the destination vectors were also verified by PCR and DNA sequencing. The forward internal (I) primers were designed to anneal within the target insert of each expression clone. The reverse

internal primers positioned within the ORF of the reporter genes were GUS R for pGWB3 or GFP R for pGWB5 (Table 3.2). The PCR program was the same used for pDONR vectors, but annealing temperatures ranged from 52℃ to 62℃, and elongation was around 45 seconds for all primer sets.

Agrobacterium infiltration of *Nicotiana benthamiana* leaves was used to test the quality of the constructs before Arabidopsis transformation. Infiltration was performed as described by Llave et al., (2000) with an additional overnight Agrobacterium incubation in *vir* induction medium (48g Na_2HPO_4, 24g KH_2PO_4, 4g NaCl, 8g NH_4Cl, 10ml 20% glucose, 10ml 1M MES pH 5.2, 1ml 0.1M Dimethoxy-4'hydroxy acetophenone, 0.1ml 1M $CaCl_2$, 2ml 1 M $MgSO_4$, in 1L). Agrobacterium culture resuspended in infiltration medium was injected into the leaf tissue with a 3cl disposable syringe.

Arabidopsis thaliana (ecotype Columbia) plants used for transformation were grown in a greenhouse at 23℃ under short day period (10h) for the first four weeks and under long day period (16h) until early flowering. Natural light and Hortilux lamps (Hortilux Super HPS LU 1000 B HTL/En) served as the light source. When plants reached the optimal stage, they were transformed with Agrobacterium containing the promoter:*GUS* and *35:ORF:GFP* constructs with the floral dipping method (Clough and Bent, 1998). To increase transformation frequencies, plants were subjected to vacuum infiltration for approximately 2 minutes at vacuum pressure of 95 kPa. Plants were covered with non-transpirable plastic, kept for 24 hrs in a dark, cool environment, and then taken to the greenhouse to be grown under standard conditions. Seeds were harvested and germinated on half-strength MS medium with 100 mg/l kanamycin sulfate. A minimum of five transformant lines per construct were generated. Transformed T_1 progenies segregating 3:1 were selected and homozygous lines were obtained through selfing.

Presence of the construct in transformed lines was verified by PCR. DNA was isolated from 100 mg tissue samples by sequential treatment with 500µl extraction buffer (200mM Tris-HCl, 250mM NaCl, 25mM EDTA, 0.5% SDS), 500µl saturated phenol, and 500µl chloroform, followed by precipitation with 400µl

isopropanol. Primers and PCR amplification program were the same used for the analysis of the purified pGWB destination vectors.

Spatial and temporal expression

To observe tissue and developmental gene expression the GUS level was assayed according to Bomblies (2002). Collected tissue was vacuum infiltrated for 30 min with a staining buffer containing 2mM of 5-bromO-4-chlorO-3-indolyl glucuronide (X-gluc) and incubated overnight at 37°C.

Influence of stress and hormones on gene expression

Transformants harboring the promoter:*GUS* constructs of At1g22400, At5g05860, and At5g05870 were subjected to heat, cold, darkness, drought, and hydrogen peroxide (H_2O_2) treatments. Effects of the hormones, abscisic acid (ABA), N^6-benzylaminopurine (BAP), gibberellic acid (GA_3) and β-indoleacetic acid (IAA) were also examined. The longer term effects were determined by GUS. Short-term responses were measured by semi-quantitative RT-PCR using wild type Arabidopsis.

Plants for short-term stress treatment were grown in sterile soil for 4 weeks. Heat (30℃), cold (10℃) and dark treatments were conducted in the growth chambers. To test the effects of drought, plants were placed on filter paper in a laminar flow hood under constant ventilation for up to 3 hours.

For short-term hormone and H_2O_2 treatments, seeds were germinated on filter paper and after a week, seedlings were acclimated in 20 ml of liquid MS media for 3 hours before starting the experiment. Seedlings were transferred to 20ml of liquid MS containing 10μM ABA, 10μM BAP, 10μM IAA or 100μM of H_2O_2. Treatments were carried out under gentle shaking to provide adequate aeration, and constant illumination for up to 3 h.

Tissue samples from all short-term experiments were collected after 0, 15, 30, 60 and 180 min, weighed, and immediately frozen in liquid nitrogen for RNA extraction (see details below). The roots of the plantlets were not included.

Total RNA was isolated using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Samples were treated with 8% (v/v) of 20% PVP and 25% (v/v) of 8M lithium chloride and incubated overnight at -20℃ whenever presence of polysaccharides or phenolic compounds was suspected. Prior to reverse transcription RNA quality was assessed with 1% agarose formaldehyde gel electrophoresis (2g agarose, 20ml 10X MOPS, 10.8ml formaldehyde, water to 200ml) and concentrations were determined and adjusted with a NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Synthesis of cDNA was achieved using the SuperScriptTM II reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA).

The resulting cDNA product was subjected to semi-quantitative PCR using elongation factor 1-α (*ef1α*; accession number X16430) as the recommended constitutive internal control under abiotic stress (Kjærsgård et al., 1997; Welinder et al., 2002; Nicot et al., 2005; Jain et al., 2006a) and band intensities were quantified by measurement of the absolute integrated optical intensity (IOD). The primers for ef1α PCR as well as those used for cDNA PCR (RT) are presented in Table 3.3. The following conditions were used for all PCRs: initial denaturation at 94℃ for 4 min followed by 30 cycles of 94℃ for 1 min, 56℃ for 45 sec, and 72℃ for 1 min. The last cycle was followed by incubation at 72℃ for 10 min.

Long-term effects were measured by relative GUS intensity. Plants were grown in soil for 4 weeks. Heat (30^{o}C), cold (10^{o}C), and darkness regimes were applied in the growth chambers. For effects of drought, plants were not watered. Leaf samples were collected daily for GUS staining, up to one week after the initiation of treatments. For the tests of growth regulators, seeds were germinated on MS medium with 1.0% (w/v) agar supplemented with 10μM BAP, 10μM GA_3, 10μM IAA, 1μM, 5μM, or 10μM ABA. Entire plants were taken 20 days after the beginning of hormone applications for GUS staining.

Table 3.2 Primer list for pDONR and pGWB PCR and sequencing.

(pDONR F and R) external primers for PCR of all donor vectors. (GUS R and GFP R) internal primers for PCR of pGWB3 and pGWB5 vectors respectively, along with (I F and R) internal primers.

Name	Sequence
pDONR F	5’-TCG-CGTTAACGCTAGCATGGATCTC-3’
pDONR R	5’-GTAACATCAGAGATTTTGAGA-CAC-3’
I At2g36750 F	5’-CGACGT-ACCTCTAGCTAAAAG-3’
I At2g36790 F	5’-GAGTATACGTCATCTTTTTAAAGG-3’
I At2g36800 F	5’-GGTGGATAAAGAAGGAGTGAAG-3’
I At1g22400 F	5’-GTG-ACGTAATTTGTGGACAAC-3’
I At5g05860 F	5’-GTCTCCATTATAGATTCTCC-3’
I At5g05870 F	5’-GTGGTTTCACTTGTCATCACC-3’
GUS R	5’-CACAAACGGTGATACGTACAC-3’
GFP R	5’-GTGGCTGTTGTAGTTGTACTC-3’

Table 3.3 Primer list for cDNA PCR quantification.

(ef1α F and R) primers for the elongation factor 1-α constitutive control. (RT F and R) amplify ORF internal regions.

Name	Sequence
ef1α F	5’-GAGGCTGCTGAGATGAACAAG-3’
ef1α R	5’-CATACCAGGCTTGATCATACC-3’
RT At1g22400 F	5’-GCGTTCCTGGCTTATCTACAC-3’
RT At1g22400 R	5’-CGGAACTCCACACGAAAGAC-3’
RT At5g05860 F	5’-GATCGATGACTGTGGATGGC-3’
RT At5g05860 R	5’-CTCCATTAACACCCTCACAGC-3’
RT At5g05870 F	5’-GCTGTGTGATCGATGATTCC-3’
RT At5g05870 R	5’-CGACCTTCCAAGTGAATCCC-3’

Cellular localization of zeatin glucosyltransferases

GFP expression of our *35S:ORF:GFP* constructs was visualized with a long-wave UV lamp (Black Ray model B 100 AP) and a 950 digital camera (Nikon, Tokyo) with UV and yellow filters. Cellular GFP localization was analyzed with a LSM 510 META confocal microscope (Carl Zeiss Inc., Thornwood, NY, USA).

Results

Prior to Agrobacterium transformation of Arabidopsis, the detection of transient expression in *N. benthamiana* leaves confirmed that both our promoter:*GUS* and *35S:ORF:GFP* fusions are functional *in planta*. All constructs displayed GUS or GFP gene expression in the abaxial epidermal cells from the infiltrated area (figures not shown).

Spatial and temporal expression

To determine the spatial and temporal expression patterns of the major zeatin O-glucosyltransferase (At1g22400), the three minor zeatin O-glucosyltransferases (At2g36750, At2g36790, and At2g36800), and the two N-glucosyltransferases (At5g05860 and At5g05870), transgenic Arabidopsis lines were generated with the promoters of the glucosyltransferase genes fused to β-glucuronidase (GUS) reporter gene. GUS expression was determined in the transformed plants at various stages of development.

At1g22400:GUS was expressed in the endosperm, which could be seen inside the seed coat as well as in emerging radicles during seed germination (Figure 3.1a). Intense GUS stain began in the root tip of young roots (Figure 3.1b), spreading over the cell division area of the apical meristem and ultimately encompassing the entire elongation zone and even beyond (Figure 3.1c). Besides the root tip, young leaf buds were also stained (Figure 3.1d). The most distinctive trait of this construct was the high expression in stomatal guard cells throughout

the plant including stems, leaves, sepals, pistils and siliques (Figures 3.1e,j). Guard cells located on the central area of the leaf blade were generally lighter (Figure 3.1g) than those closer to the hydathodes at the leaf edge (Figure 3.1h), an area rich in vascular bundles.

At2g36750:GUS was not expressed in germinating seeds and roots (Figures 3.2a,b), but was expressed in vascular tissue of leaves, sepals and petals, the stigma, and the abscission zones of the siliques (Figures 3.2d,f). At2g36790:GUS presented a somewhat similar pattern, although the GUS stain was stronger and more limited to the vasculature throughout the aerial part of the plant, accumulating on bundle junctions, the leaf apex, sepals, petals, and pistil (Figures 3.3c,f).

At2g36800:GUS expression was detectable at early stages of seed germination in the endosperm but was absent in emerging radicles (Figure 3.4a). In developing roots, staining began at the zone of cell elongation on the upper margin of the root apical meristem. As the root grew older, GUS activity spread upwards until the whole length of the root, including root hairs, was stained. When spreading towards the root apex At2g36800:GUS expression eventually reached the area of cell division, leaving only the root cap unstained (Figures 3.4b,c). GUS stain was light in hypocotyls (Figure 3.4c). Young leaf buds also lacked staining, but with growth, expression increased in mesophyll cells (Figure 3.4c) although it decreased as leaves matured (Figure 3.4d). Staining of reproductive organs was located in flower sepals, older pedicels and remnant of the style but not the stigma (Figures 3.4e,f).

At5g05860:GUS was highly expressed in meristematic tissue of emerging radicles (Figure 3.5a), cell division area of the root tips (Figure 3.5b), vascular tissue of seedlings, mesophyllic tissue of cotyledons, young leaf buds (Figure 3.5c) and somewhat less in older leaves (Figure 3.5d). Expression was also present in flower sepals, stigmas, anther filaments, and very intensely in the abscission zones of the siliques (Figures 3.5f,g). A distinguishing trait of this

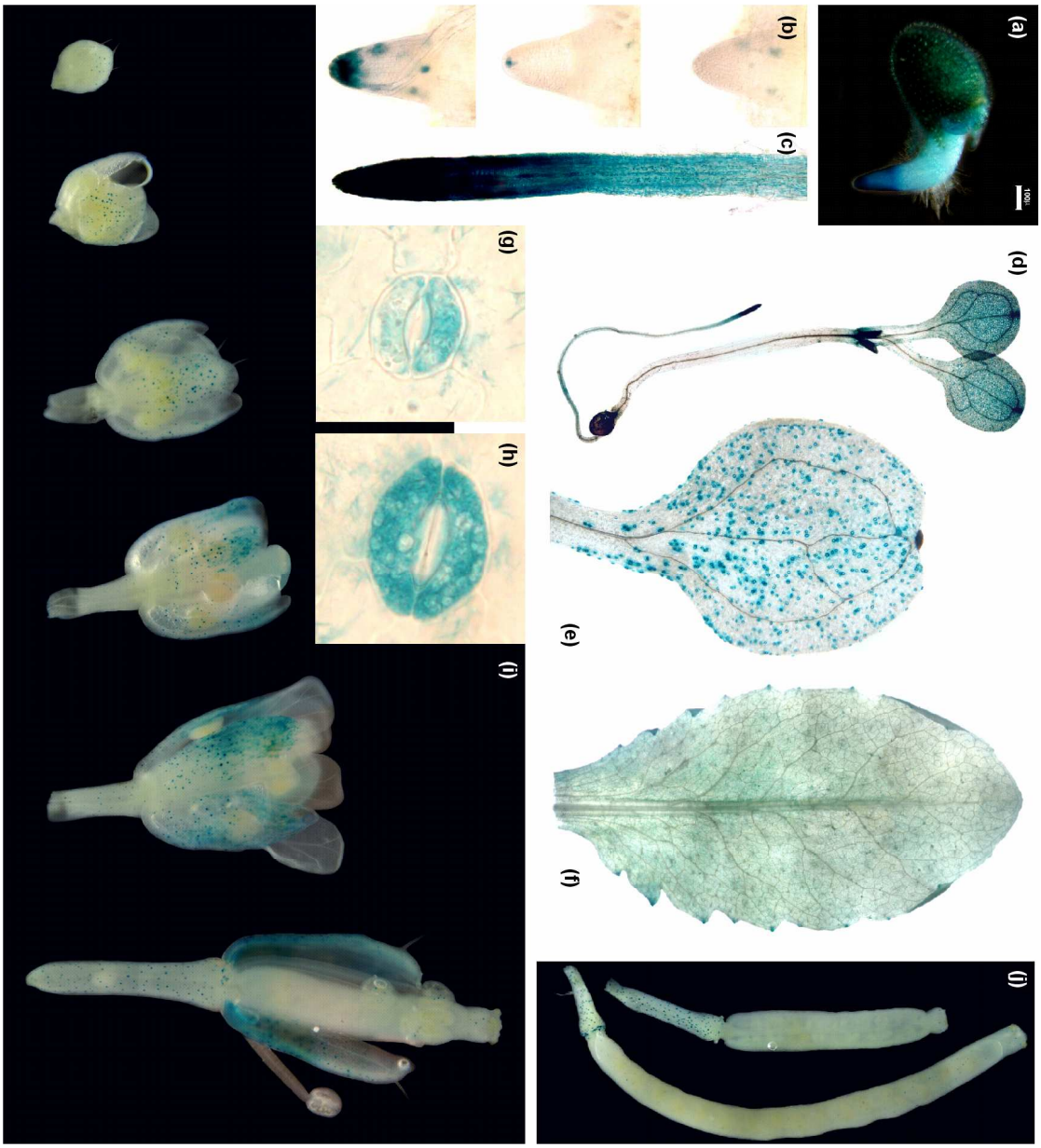

Figure 3.1 Promoter:*GUS* expression of At1g22400.

GUS stain is present in: (a) endosperm and the radicle tip of germinating seedling; (b) cell division area at the apex of emerging roots (c) whole apical meristem of older roots; (d) root tips, young leaf buds and stomatal guard cells of one-week-old plantlets; (e) young cotyledon; (f) mature rosette leaves; (g) guard cell at central leaf blade; (h) guard cell near a hydathode; (i) guard cells of flowers, sepals, pistils; and (j) siliques.

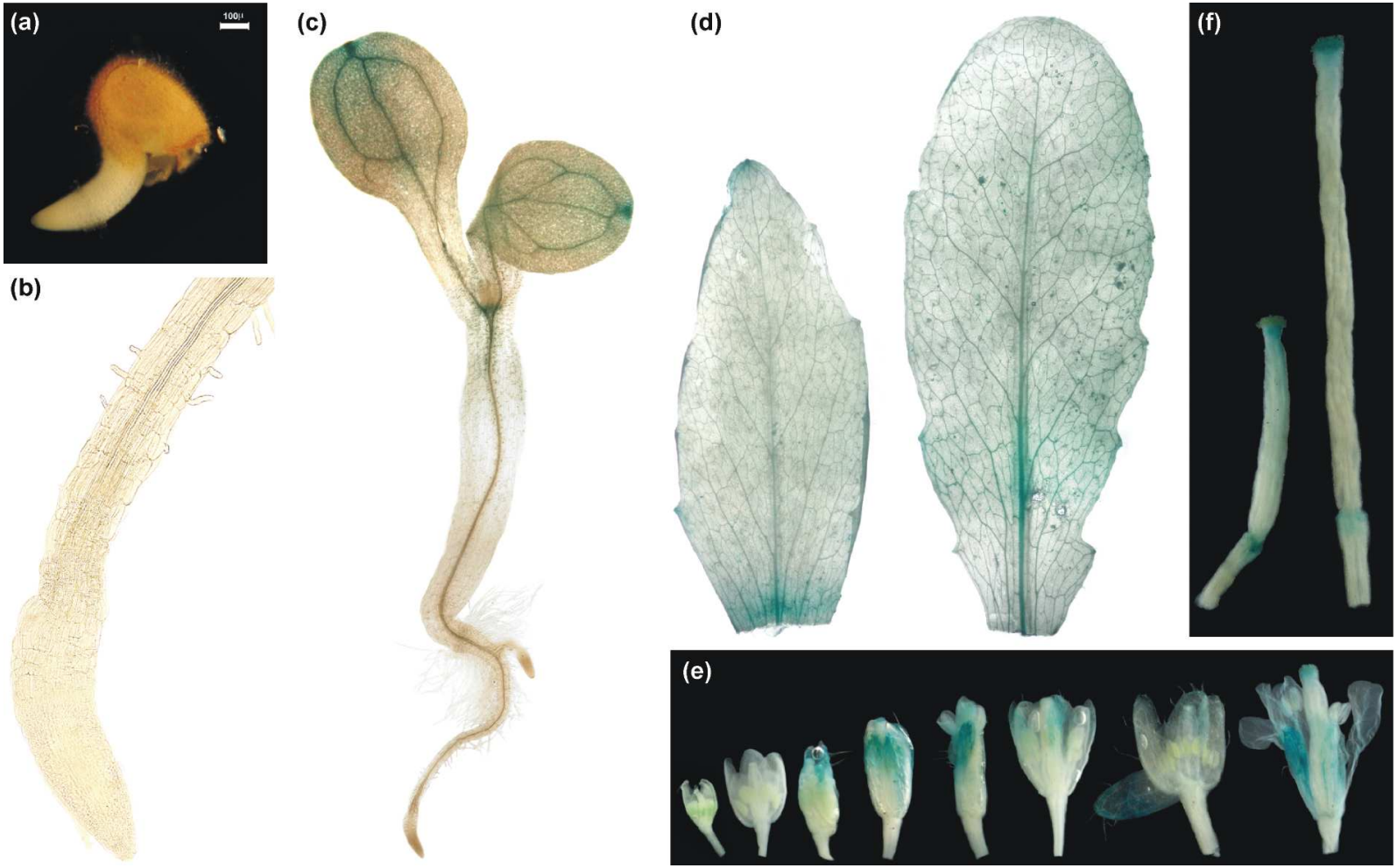

Figure 3.2 Promoter:*GUS* expression of At2g36750.

GUS stain is absent in (a) germinating seeds and (b) roots but present in (c) vascular tissues of the hypocotyl and leaves of one-week-old plants; (d) veins of leaves; (e) sepals, petals, stigma; and (f) the abscission zones of siliques.

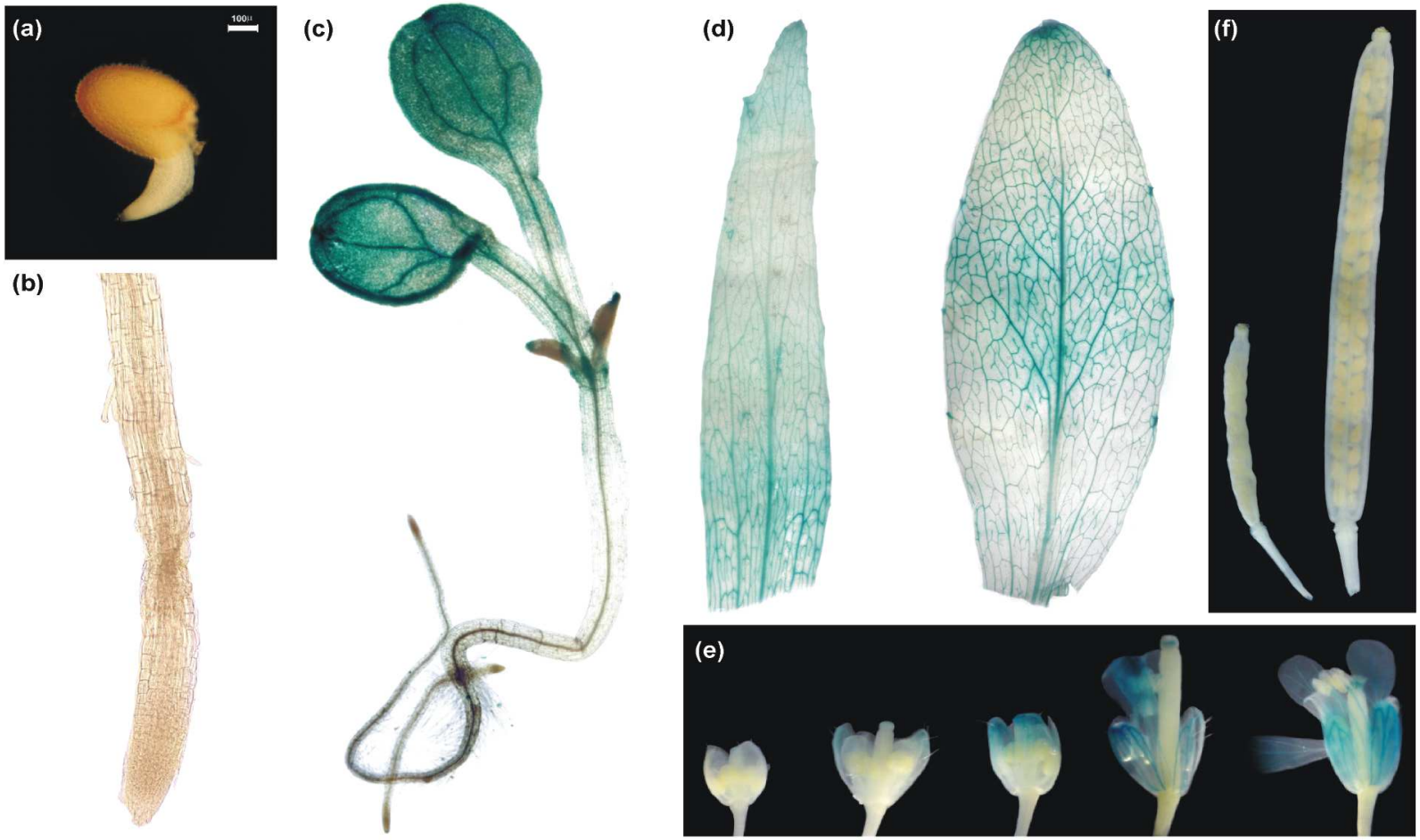

Figure 3.3 Promoter:*GUS* expression of At2g36790.

GUS stain is absent in (a) germinating seeds and (b) roots; present in (c) vascular tissues of hypocotyls of one-week-old plantlets; (d) the apex and bundle junctions of leaves; (e) flowers, sepals, petals and the pistil; and absent in (f) siliques.

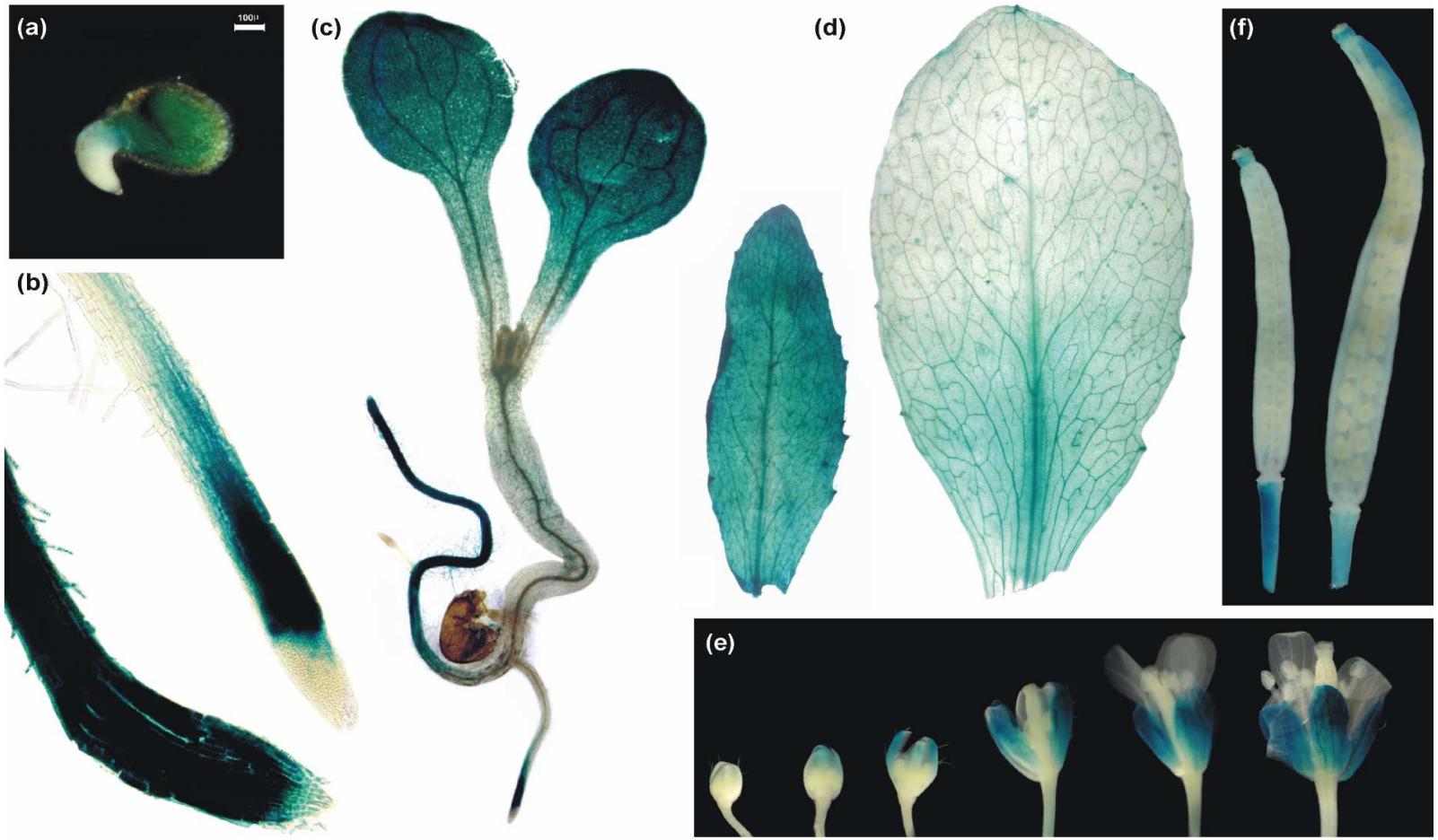

Figure 3.4 Promoter:*GUS* expression of At2g36800.

GUS stain is present in (a) endosperm but not the radicle of germinating seed; (b) in roots but not in the root cap; (c) cotyledons and vascular tissue of the hypocotyls; (d) mesophyllic and vascular tissues of young and mature leaves, high near the hydathodes; (e) sepals of flowers; (f) pedicels of siliques and remnants of the style but not the stigma.

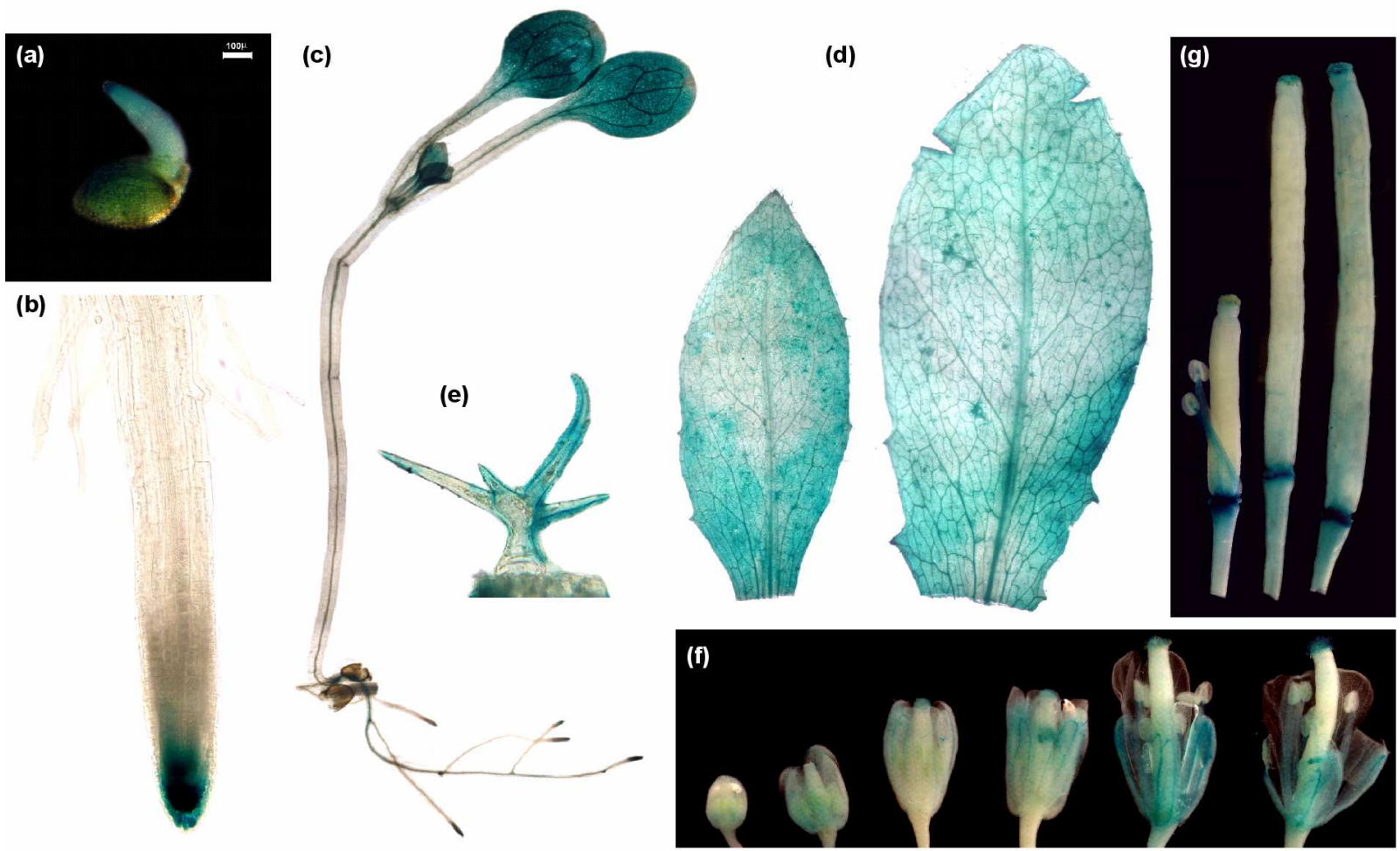

Figure 3.5 Promoter:*GUS* expression of At5g05860.

GUS stain is present in (a) the radicles of germinating seed; (b) the root meristem; (c) root tips, hypocotyl, leaf buds and cotyledons of one-week old plantlets; (d) vascular bundles of leaves; (e) trichomes; (f) sepals, stigmas, and anther filaments; and (g) abscission zones of the siliques.

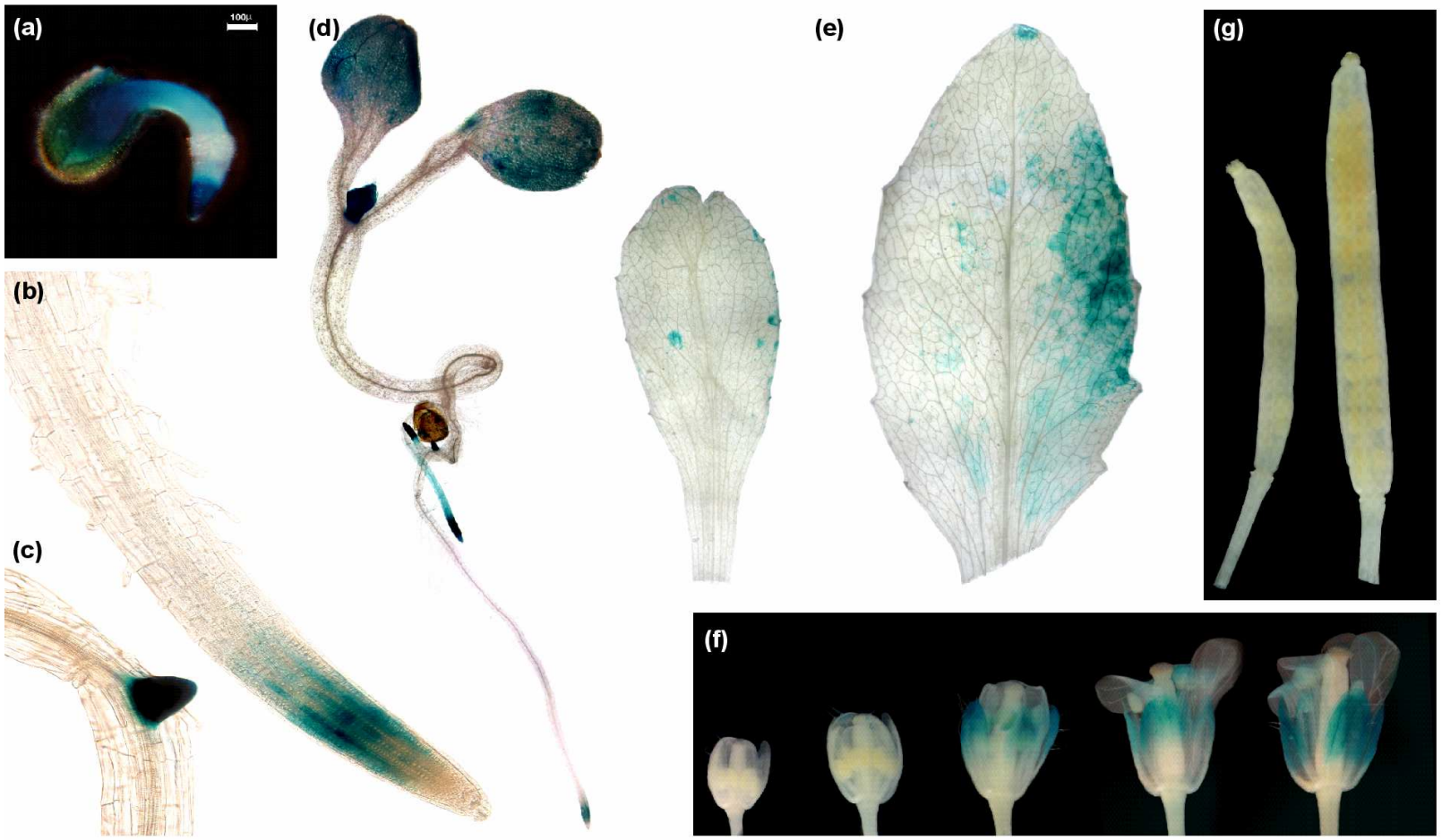

Figure 3.6 Promoter:*GUS* expression of At5g05870.

GUS is present in (a) endosperm and the radicle of germinating seed; (b) the elongation zone of roots; (c) emerging lateral roots (d) root tips, leaf buds, and cotyledons of one-week-old plantlets; (e) leaves (inconsistently); (f) sepals; but not in (g) siliques.

construct was the expression in all trichomes throughout the plant including the flower sepals (Figure 3.5e).

At5g05870:GUS expression occurred in the endosperm and in radicle tips of germinating seeds (Figure 3.6a). In roots, GUS activity began at the cell elongation area immediately above the apical meristem and spread towards the root apex (Figure 3.6b). Lateral roots were more stained than the main root but followed the same temporal pattern (Figures 3.6c,d). This root expression pattern resembles that of At2g36800:GUS. Staining was abundant in cotyledons and young leaf buds (Figure 3.6d), but less intense and more blotchy in older leaves (Figure 3.6e). As to reproductive structures, only the sepals were stained and siliques did not show any GUS expression (Figures 3.6f,g). Except for At1g22400, none of the genes were expressed in the guard cells.

Influence of stress and hormones on gene expression

Short-term regulation of At1g22400, At5g05860, and At5g05870 expression by abiotic stress and plant hormones was determined by semi-quantitative RT-PCR. Overall, these factors had small effects on gene expression. Drought stress had the strongest effect, enhancing transcription of At1g22400 and At5g05860 (Figure 3.7a). The effect on transcription of At1g22400 was most striking, increasing within 15 minutes and to a five-fold after 3 hours. ABA also affected expression of At1g22400, increasing three-fold after 3h (Figure 3.7b). BAP did not have any consistent effects except for the increase in At22400 after 3h (Figure 3.7c). Diurnal fluctuations of gene expression were small and transitory, generally increasing within 15 to 30 min after day/night transitions, followed by a return to base level after 3 or 6 hours (Figures 3.7d,e,f). None of the other BAP, IAA or H_2O_2 treatments had a consistent and significant effect on mRNA levels.

Long-term effects were examined through GUS staining of transformants containing promoter:*GUS* constructs. This allowed evaluation of intensity as well as distribution of gene expression. At1g22400:GUS activity was significantly

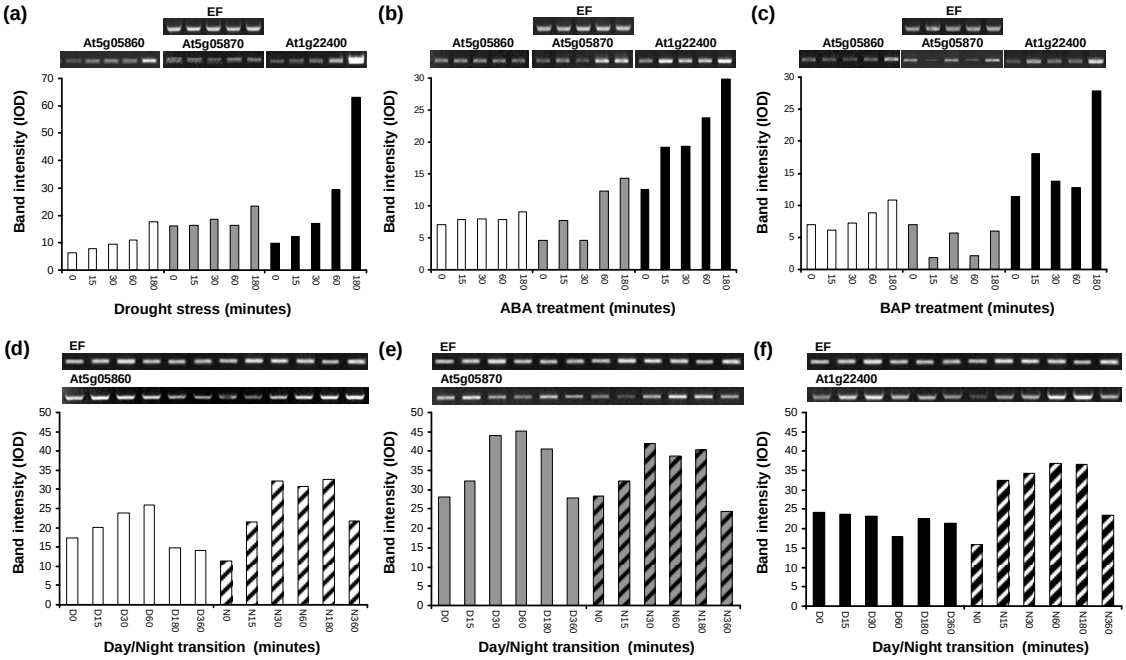

Figure 3.7 Effect of short-term treatments on mRNA (RT-PCR) levels.

At5g05860, At5g05870 and At1g22400 cDNAs were obtained from wild-type Arabidopsis ecotype Columbia. Effects of (a) drought, (b) ABA, (c) N^6-benzylaminopurine, and (d,e.f), light and dark. Elongation factor ef1α (EF) was used as the constitutive internal control. Band intensity is quantified by measurement of the absolute integrated optical intensity (IOD). The bar figure gives the average of two experiments.

increased by drought and heat (Figure 3.8a,b). GUS consistently accumulated in the guard cells and then spread to surrounding tissue (Figure 3.8a,b). Although guard cells closer to hydathodes are shown in Figure 3.8, GUS intensity also increased in guard cells closer to the midrib of leaves. Conversely, darkness inhibited GUS activity (Figure 3.8c). Although less distinctively expressed, other genes showed similar changes in GUS expression in response to stress. At5g05870:GUS was also induced by drought and heat, accumulating on vascular and mesophyllic tissue around the central leaf vein. Because the staining levels of At5g05870:GUS was already lower than those of At1g22400:GUS, any decrease caused by darkness were undetectable. Cold was the only stress-related factor tested that had no effect on any of the lines.

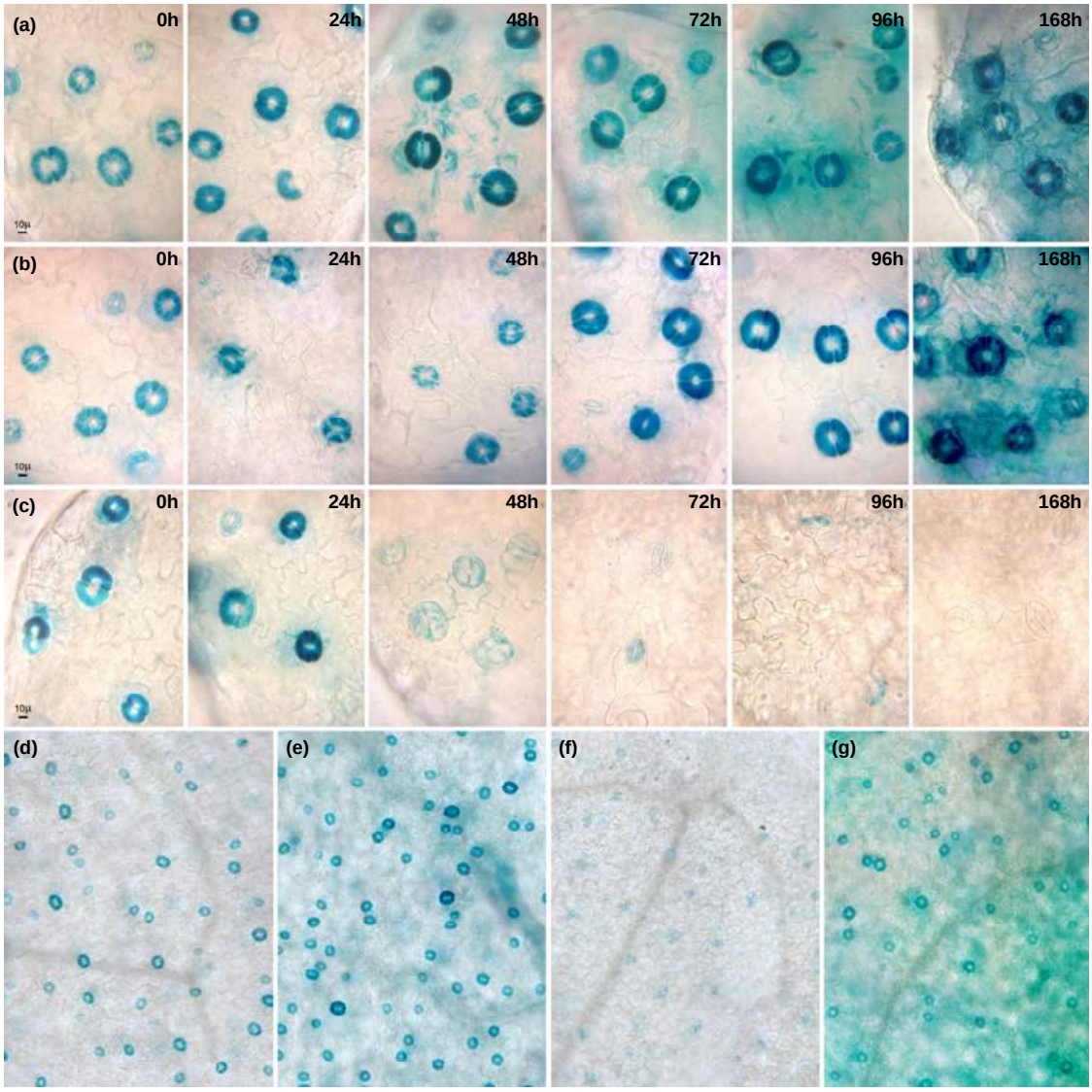

Figure 3.8 Effect of long-term treatments on At1g22400 expression.

Visualized by GUS stain of promoter:*GUS* transformants. (a) drought; (b) heat; (c) darkness; (d-f) after 20-d hormonal treatments: control (d), 10μM ABA (e), 10μM BAP (f) and 10μM IAA (g). The pictures present guard cells near the hydathodes.

Besides inducing morphological changes, long-term hormonal treatments altered relative GUS expression levels among different tissue types. Treatment with ABA enhanced guard cell expression of At1g22400 (Figure 3.8d,e) and also mesophyllic expression of all lines. BAP treatment decreased staining of guard

cells of At1g22400:GUS (Figure 3.8f), but a significant GUS increase in meristematic cells of both At1g22400:GUS and At5g05870:GUS. Young shoots and roots appeared darker than controls. Treatment with IAA enhanced mesophyllic expression of At1g22400:GUS, but left guard cells unaffected (Figure 3.8g). IAA did not cause significant changes in other lines. Application of GA_3 did not induce any changes other than stem elongation, and H_2O_2 had no significant effects.

Cellular localization of zeatin glucosyltransferases

These six proteins do not contain any signal sequences for cellular location, which is generally the case with UGTs belonging to Family 1 (Li et al., 2001), leading to the supposition that they are located in the

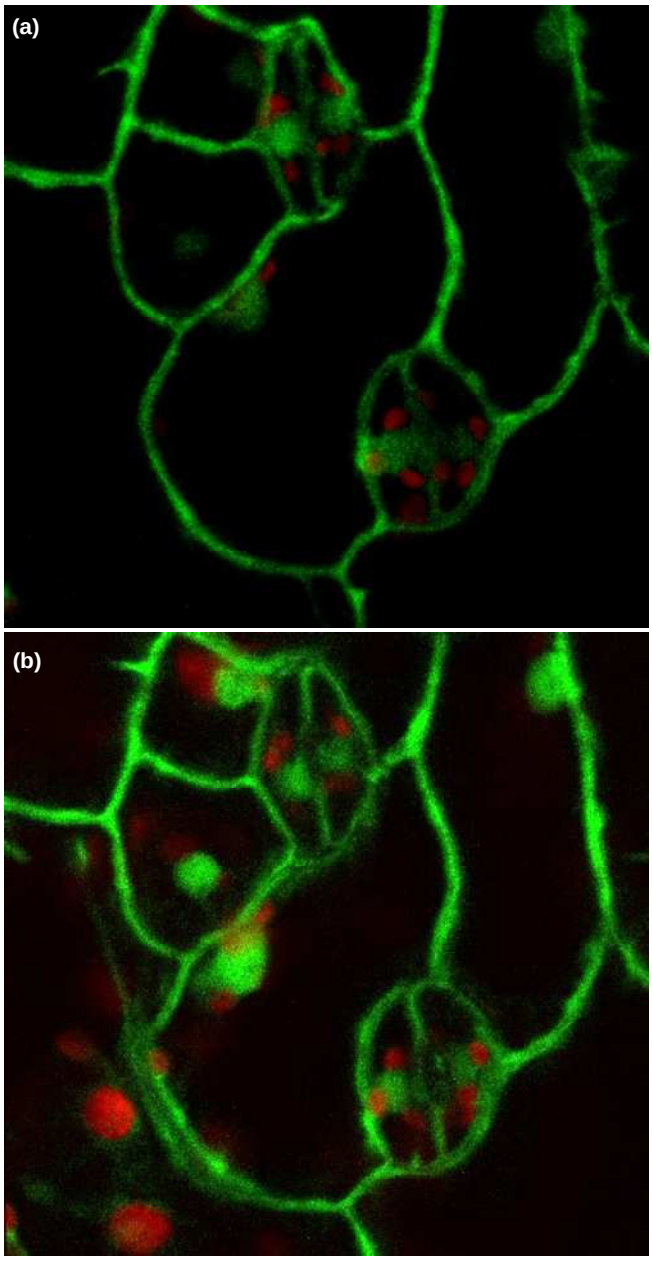

Figure 3.9 Subcellular localization of At5g05860:GFP fusion protein.

At5g05860, At5g05870 and At1g22400 protein fusions accumulated in the cytoplasm pressed between the cell wall and vacuoles, in trans-vacuolar strands, and in nuclei (a,b). Confocal cross sections confirm ORF:GFP fusions are localized inside the nuclei.

cytoplasm. To substantiate this, the locations of the major glucosyltransferases, At1g22400, At5g05860, and At5g05870, were determined in plants transformed with constructs containing the 35S promoter and the ORFs of the genes with the green fluorescent protein (sGFP) gene fused at the C-terminus. All three proteins were localized in the cytoplasm, trans-vacuolar cytoplasmic strands and nucleus (Figure 3.9).

Analysis of promoter sequence motifs

Primers for the promoter:*GUS* constructs were designed to amplify the entire putative promoter regions plus the 5' UTR of all six glycosyltransferases. Because the exact length of those promoter regions is not known, our primers included the complete upstream sequence up to the adjacent gene in order to ensure inclusion of any activator or enhancer elements into the inserts.

Motif analyses with the signal scan program from the PLACE database of Plant *Cis*-acting Regulatory DNA Elements (Prestridge, 1991; Higo et al., 1999) revealed several regulatory motifs in all six genes, but their high frequency indicated that mere presence of such elements may not necessarily imply any relevant function. Elements may need to occur in close repeats or at the correct location. For instance, the stomatal location motif AAAAG and the dehydration-responsive element GTCAC occurred several times in all upstream regions, but these or other *cis*-acting elements or location motifs were never seen as close repeats in the same upstream region except for At1g22400, which contains several copies of different regulatory sequence motifs, all related to senescence and water stress transcription signaling (Figure 3.10). These motifs are diverse in nature, but all function as target sites for transcription factors:

The first group of sequences share a G-box ACGTG core motif known as ABA Response Elements (ABREs). Upstream of At1g22400 are two copies of the motif, although based on comparison with promoters from other ABA-inducible genes (Choi et al., 2000; Nakashima et al., 2006), the ABRE located 186bp upstream of the translation start is most likely the functional regulator. A second

type of *cis*-acting element present only upstream of At1g22400 is the dehydration-responsive E-box CATGTG motif, a recognition site for MYC-like transcriptional activators. The MYC element is repeated twice, but only the first -423bp motif is at a relevant distance from the ATG (Simpson et al., 2003). The third element group is the TAAAG sequence motif, a target site for trans-acting Dof transcription

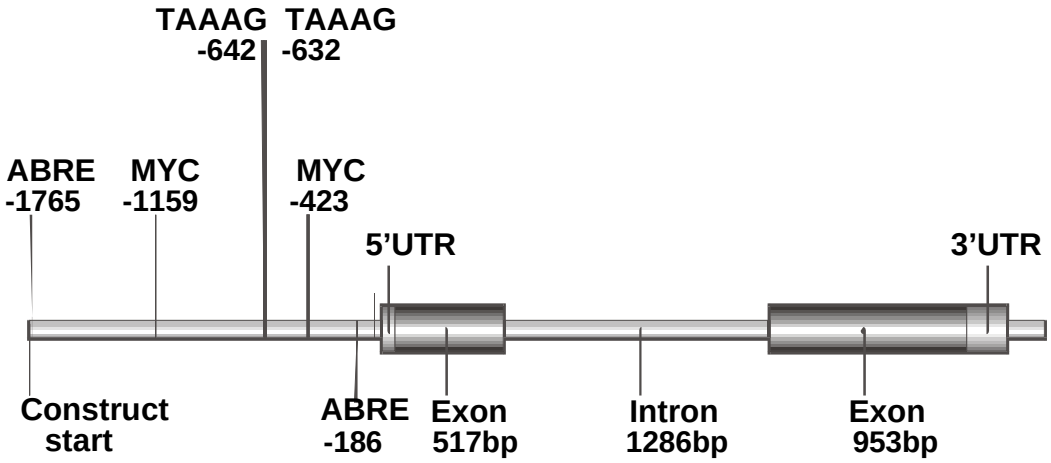

Figure 3.10 Analysis of upstream sequence motifs of At1g22400.

Positions relative to the ATG codon. (ABRE) ABA-responsive G-Box ACGTG sequence; (MYC) dehydration-responsive E-box CATGTG motif, a recognition site for MYC-like transcriptional activators; (TAAAG) a target site for Dof transcription factors controlling guard cell-specific gene expression.

factors controlling guard cell-specific gene expression (Plesch et al., 2001). Upstream of At1g22400 are two tandem repeated TAAAG motifs. The occurrence of multiple copies is known to enhance Dof affinity and therefore stomatal specificity (Yanagisawa and Schmidt, 1999). Other minor binding motifs were identified; including NGAAT, CAAT box1 and GATA box, all related to ARR transcriptional activators for cytokinin signaling (Kusnetsov et al., 1999; Sakai et al., 2000; Lee et al., 2006).

Discussion

Glucosyltransferase activity in cytokinin biosynthetic organs

Promoter:*GUS* transgenic plants were used to determine expression patterns of major genes encoding zeatin glycosyltransferase. The most relevant genes are the O-glucosyltransferase At1g22400 and the two N-glucosyltransferases, At05g05860 and At5g05870. These three genes have different patterns of expression, but in general are expressed in germinating seeds, root tips, young leaves, and the abscission zone of fruits. These are also sites of cytokinin biosynthesis, as supported by classical studies (Letham, 1994) and expression of isopentenyltransferases (IPTs) and other biosynthetic genes (Miyawaki et al., 2004; Takei et al., 2004b; Takei and Sakakibara, 2005). Therefore, formation of both the reversible (O-) or the irreversible (N-) glucosides must be involved in regulating the appropriate levels of active cytokinins close to the biosynthetic sites. While O-glucosyltransferases may serve to temporarily sequester cytokinins, expression of N-glucosyltransferase genes at biosynthetic sites indicates that the level of biosynthesis exceeds the requirement for cytokinins. Also expression of some of the cytokinin oxidase/dehydrogenase (CKX) genes was induced by exogenously or endogenously enhanced cytokinin synthesis (Chatfield and Armstrong 1986; Kamínek and Armstrong 1990; Zhang et al., 1995; Redig et al., 1997; Motyka et al., 2003) or directly linked to cytokinin biosynthetic sites (Schäfer and Schmülling, 2002; Yang et al., 2002; Werner et al., 2003; Brugière et al., 2003). One of the CKX functions proposed by Werner et al., (2003) concerns adjustment of the natural fluctuations of cytokinin levels (Redig et al., 1996; Nováková et al., 2005). Similarly, cytokinin glucosylation may be involved in adjusting cytokinin levels during cell division, with the advantage of being reversible in the case of O-glucosylation. However, the problem is likely very complex, with rapid cellular fluctuations and compartmentation of both cytokinin metabolites and the enzymes involved (Fußeder and Ziegler, 1988; Dean et al., 2003; Schmülling et al., 2003; Kasahara et al., 2004; Sakakibara,

2006), and may be different for each organ/tissue. More precise methods including precisely targeted cytokinin and enzyme measurements will be required to solve this problem.

Glucosyltransferase expression and vegetative development

Expression of At1g22400 and A15g05860 is high in the division zone while that of At5g05870 is highest in the elongation zone. The latter may serve to adjust cytokinin levels after transition from cell division to elongation. The former two may be involved in adjusting cytokinin levels during cell division, as described above. Overexpression of both the *ZOG1* gene in maize (Chapter 2) and *CKX* genes in Arabidopsis (Werner et al., 2003) increased root mass. While Werner et al., (2003) concluded that cytokinins are negative regulators of root development, we take the view that the cytokinin levels are higher than required for optimal root growth. Further lowering of root cytokinin levels by increasing cytokinin GT or CKX activity, however, would compromise shoot growth, as demonstrated by the decrease in plant size in the *Ubi:ZOG1* maize plants described in Chapter 2.

The N-glucosyltransferase genes are highly expressed in cotyledons and young leaf buds. The connection between expression in leaf buds in relation to sites of cell division has been described in an earlier section. Also expression of At1g22400:GUS is high in young buds, but the expression in mesophyll cells ceases early on. Gene expression occurs consistently in stomatal guard cells, indicating that there is a need for lowering of active cytokinin levels in these cells.

A possible role for zeatin O*-glucosylation in stomatal control*

The expression of At1g22400 in guard cells raises the question of whether O-glucosylation of zeatin plays a role in stomatal control. Plant hormones are known to be important factors in controlling stomatal movement (Schroeder et al., 2001). Of these, the most extensively studied and best understood hormone is abscisic acid (ABA). ABA accumulates rapidly in guard cells in response to water

deficit, bringing about stomatal closure. In contrast, cytokinins cause opening of stomates and their levels decrease during water deficit (Pospíšilová et al., 2000). Generally, the ABA/cytokinins ratio seems to be a strong determinant of stomatal aperture (Fußeder et al., 1992). The RT-PCR analyses of At1g22400 have shown that the same drought, heat, and ABA treatments known to induce stomatal closure, also rapidly upregulate At1g22400. These results are further supported by the presence of guard cell-specific, ABA-responsive, and dehydration-responsive motifs in the upstream regions of this gene. This observation argues for involvement of zeatin O-glucosyltransferase in stomatal movement.

While no previous research has established a link between cytokinin glucosylation in guard cells and stomatal movement or respiration, there is some indication that cytokinin glucosides may be involved in long-distance water stress signaling. Xylem sap of water-stressed tomato contained only one-half the O-glucoside levels of non-stressed controls (Davies et al., 2005). Furthermore, application of O-glucosides via the xylem resulted in significant increases of transpiration in derooted oat and wheat (Badenoch-Jones et al., 1996), showing that xylem sap derived glucosides can contribute to the active cytokinin pool in the leaves, most likely through the action of cytokinin-specific *β*-glucosidases such as those identified in maize and *Brassica napus* (Brzobohatý et al., 1993; Falk and Rask, 1995). Recent studies with transgenic tobacco overexpressing cytokinin *β*-glucosidases or O-glucosyltransferases showed the intertwined effect of those enzymes on O-glucoside levels *in planta* and their differential compartmentation within leaf cells (Polanská et al., 2007).

A clearer connection has been established, however, between levels of glucosides of ABA and water stress. ABA glucosides are known to be formed as a response to drought and transported through the xylem as part of a root-to-shoot stress signal (Xu et al., 2002; Sauter and Hartung, 2000; Sauter et al., 2002). Upon arrival in the leaf apoplast, the glucosides can be converted to free ABA by *β*-glucosidases (Dietz et al., 2000; Lee et al., 2006). In the leaf, ABA induces stomatal closure and expression of stress-related genes such as CKXs (Brugière et al., 2003). Similarly to the drought/ABA-induced regulatory pathway, At1g22400

could be part of a system controlling guard cell aperture under water stress by reducing levels of active cytokinins.

Glucosyltransferase expression and reproductive development

The most evident feature of the GUS staining patterns of At5g05860 and At5g05870 was the very intense stain of the fruit abscission zone of At5g5860. It is likely that the glucosyltransferase activity serves to modulate cytokinin activity in this biosynthetic site (Brugière et al., 2003). In addition, it is well known that production of ethylene, a stimulator of abscission, is induced by cytokinin (Lau and Yang, 1973; Lau et al., 1977; Yu et al., 1981). By lowering cytokinin levels, O-glucosylation could serve to decrease ethylene production, thereby delaying silique abscission. GUS stain was negligible in flowers and fruits of At1g22400:GUS except for the obvious presence of GUS in guard cells of sepals and pedicels, indicating universal expression of At1g22400 in guard cells.

Expression of all three genes occurred in the seed endosperm. This observation is in agreement with our earlier Northern analyses of *ZOG1* and *ZOX1*, which showed high activity in endosperms (Martin et al., 1999a,b). Cytokinins are known to be synthesized in the embryo and transported to the emerging cotyledons (Gepstein and Ilan, 1979; Gepstein and Ilan, 1980; Ilan and Gepstein, 1981; Muñoz et al., 1990). It has been suggested they may assist redistribution of storage protein reserves during radicle and cotyledon growth. We propose that glucosyltransferases may store O-glucosides in the endosperm in order to ensure an adequate supply of active cytokinins during seedling development.

Subcellular localization of glucosyltransferases

The *35S:ORF:GFP* lines show fluorescence in the cytosol, trans-vacuolar cytoplasmic strands, and nuclei. The presence of fluorescence in the cytoplasm is expected (Li et al., 2001; Ross et al., 2001; Bowles et al., 2006), but occurrence in

the nuclei is puzzling. The average size of our *ORF:GFP* protein fusions is 79kDa, which exceeds the size limit for protein diffusion across nuclear pores (≤40kDa) proposed by Görlich and Mattaj (1996). Protein subcellular localization of our glycosyltransferases prediction with the LOCHom and PSORT databases (Nair and Rost, 2005; Horton et al., 2006) did not find any nuclear localization signal (NLS). There are many examples of nuclear-localized proteins that may be imported into the nucleus through NLS-independent mechanisms (e.g., Fagotto et al., 1998; Amador et al., 2001; Li et al., 2003; Kong et al., 2006; Verslues et al., 2006), but these are not related to the UGTs. However, immunolocalization (using a monoclonal antibody to this protein) detected *ZOX1*, another zeatin glycosyltransferase, in the nucleus (Martin et al., 1993).

Significance of the minor O*-glucosyltransferases*

The minor zeatin O-glucosyltransferases, At2g36750, At2g36790, and At2g36800, have only a fraction (1-3%) of the specific activity of At1g22400 for zeatin (Hou et al., 2004; Martin et al., unpublished results). However, At2g36800 has seven-fold higher specific activity for dihydrozeatin than zeatin (Hou et al., 2004). Since these enzymes have other major substrates (Jones et al., 2003; Poppenberger et al., 2005), their relevance to cytokinin metabolism is in question. The affinities (K_m values) of these enzymes for cytokinins and their other substrates are not known, nor are the concentrations of the various substrates in plant tissues. Thus it is difficult to predict whether zeatin or any other cytokinin competes well against the other substrates. Yet, At2g36800 is highly expressed in organs with high cytokinin activity such as root tips, cotyledons, and young leaves. Interestingly, At2g36800 is also expressed in the remnant of the style but not the stigma and in the pedicel but not the abscission zone (Fig. 3.4f). This expression is the opposite of At5g05870, indicating complementary expression. The high expression of At2g36800 in relevant tissues may result in some activity of the enzyme *in planta* with zeatin or dihydrozeatin as substrate, despite competition for binding to the enzyme from brassinosteroids, compounds

occurring at low concentrations in plant tissues. This is an example of the general and complex problem of enzyme promiscuity, where several substrates may compete and success depends on a number of parameter such as substrate concentrations, enzyme affinities, and specific activities towards each substrate.

Conclusions

The expression patterns of the O- and N-glucosyltransferases of Arabidopsis support a role for glucosylation in cytokinin homeostasis in various plant organs. Cytokinin glucosylation and biosynthetic sites often overlap, suggesting a function in modulating active cytokinin levels and in storing cytokinins for later use. Additional functions may depend on the site of expression. Expression of At1g22400 in stomata, together with the effects of ABA and drought, may imply a function of this gene in regulating stomatal movement and transpiration. Studies with At1g22400 knock-out mutants could provide further support for such functions.

CHAPTER 4

GENERAL CONCLUSION

This study focused on the effects of cytokinin glucosylation on plant development and the regulation of glucosyltransferase gene expression, both contributing to our understanding of the function of cytokinin O-glucosyltransferases. Major findings, problems, and some remaining questions are discussed in this chapter.

Effects on plant growth and development

With regard to vegetative growth, overexpression of *ZOG1* in maize causes a decrease in shoot meristem size, which in turn results in thinner stems and reduced plant size. Although it is impossible to accurately measure cytokinin levels in meristems, the root cytokinin analyses revealed slightly lower cytokinins in transformants than controls, indicating that the smaller meristems are related to a decrease in active cytokinins. The importance of cytokinin glucosyltransferases in root and shoot meristem development is supported by the Arabidopsis glucosyltransferase gene expression patterns in roots, suggesting that GTs may regulate meristem growth by adjusting cytokinin levels during cell division (At1g22400 and At5g05860) and assist the transition to cell differentiation (At5g05870). Cytokinin glucosyltransferase activity at early stages of vegetative differentiation is likely to determine plant architecture throughout the life cycle. However, the higher leaf chlorophyll levels and delayed senescence in transformants are traits indicative of high cytokinin (Richmond and Lang, 1957; Smart et al., 1991; Li and Guilfoyle, 1992; Gan and Amasino, 1995; Robson et al., 2004) and indeed, the cytokinin levels were elevated in leaves. The increase of active cytokinins could be due to *de novo* biosynthesis by IPTs or release of active aglycones by *β*-glucosidases. Since subcellular compartmentation studies

of IPTs placed the first step of *de novo* cytokinin biosynthesis in chloroplasts (Kasahara et al., 2004; Sakakibara, 2006) and cytokinins are known to stimulate photosynthesis, chloroplast protein formation and replication (Van Staden et al., 1988; Zavaleta-Mancera et al., 1999), higher levels of cytokinins in those organelles may bring about the increases in chlorophyll levels. This, however, assumes direct action of cytokinins in chloroplasts, which has never been demonstrated.

As to reproductive development, the feminization of homozygous *Ubi:ZOG1* tassels is most interesting. Although cytokinins are known to influence sex expression, they have never been linked to tasselseed formation. Whether tasselseed occurrence is related to lower or increased cytokinin levels is difficult to determine since any changes in development are bound to bring about changes in cytokinin levels and composition, making it impossible to distinguish between cause and effect. The smaller tassels of hemizygous plants are indicative of lower cytokinins. However, the tasselseeds occurred parallel to higher chlorophyll and delayed senescence and thus it is also plausible that tasselseed formation is associated with increased cytokinin levels or particular cytokinin metabolites. Moreover, delay of senescence and feminization proved sensitive to light intensity and/or quality; setting them apart from all other observed *Ubi:ZOG1* traits. Thus it remains unknown how *ZOG1* overexpression leads to an increase in leaf cytokinin levels or how cytokinins are involved in tasselseed formation.

Expression of glucosyltransferases in growing tissues

Cytokinin glucosylation was often detected in growing tissues, which are also the main sites of cytokinin biosynthesis. Thus it is likely that GT expression takes part in the regulation of cytokinin homeostasis at these sites. Irreversible N-glucosylation may serve to modulate excess of active cytokinins, sharing a common role with degradative cytokinin oxidase/dehydrogenase (CKX) genes. Whereas the primary role of O-glucosylation would also be stage-specific

regulation of active aglycone levels, its influence is potentially broader and far-reaching since O-glucosides can be translocated and possibly stored as a pool of reserve cytokinins until favorable conditions induce their reactivation by *β*-glucosidases. Consequently, O-glucosyltransferases would have the potential to affect organs and tissues that are sinks for cytokinins and O-glucosides may constitute a long-distance and long-term communication system.

An important but very complex issue is the distribution of cytokinin metabolites and enzymes in cellular compartments, which is relevant both to channeling cytokinins to the xylem sap and their use upon arrival in the sink tissues. Whereas the tissue and cellular location of enzymes can be determined through the use of reporter genes such as GUS and GFP, the location of cytokinin metabolites in tissues and within cells is uncertain since the current analytical methods are inadequate for such precise determinations. For instance, cytokinin biosynthesis takes place in the plastids (Kasahara et al., 2004; Sakakibara, 2006) and also the maize *β*-glucosidase is located in plastids (Kristoffersen et al., 2000), whereas the Arabidopsis cytokinin glucosyltransferases are located in the cytoplasm and nucleus. It is unclear whether or where O-glucosides coming from the xylem sap as part of the root-to-shoot communication system are cleaved to the aglycones by *β*-glucosidase. Nor is it known whether the pool of glucosides stored in the vacuoles (Fußeder and Ziegler, 1988) can ever be retrieved. However, glucosides can gain access to membrane-bound transporter systems (Bowles et al., 2006), implying that trans-membrane transport could take place. Thus although it is apparent that IPTs together with metabolic enzymes such as O-glucosyltransferases, CKXs, and *β*-glucosidases regulate the proper amount of cytokinin needed for growth, the exact ways in which the various pathways interact and the significance of the intra-cellular locations of the enzymes remain elusive.

A possible role for zeatin O-glucosylation in stomatal control

One possible target of cytokinin GT action was uncovered by the expression of the cytokinin O-glucosyltransferase At1g22400 in guard cells. In contrast with the more general localization of other glucosyltransferases, the expression of At1g22400 almost exclusively in one cell type of the stems, leaves, and flowers calls for speculations on its possible role. One possible explanation is that expression of At1g22400 in the stomata simply answers the need for lowering active cytokinin levels in these cells. Promoter-reporter studies with Arabidopsis cytokinin oxidase/dehydrogenases (*AtCKX4* and *AtCKX6*) showed similar guard cell expression patterns (Werner et al., 2003). Furthermore, there is evidence that CKXs can be induced by stress and ABA (Brugière et al., 2003), in a similar fashion to At1g22400. These data would support a parallel role of CKXs and cytokinin O-glucosyltransferases in short-term ABA-mediated decrease of cytokinins to facilitate guard cell closure.

A more intricate function may stem from the reversibility of O-glucosylation. Cytokinin O-glucosides generated by At1g22400 activity may be hydrolyzed by *β*-glucosidases when the need for more aglycone arises. There is a requirement for rapid signaling in the regulation of guard cell movements (Schroeder et al., 2001). Our short-term RT-PCR experiments confirm the quick response in expression of At1g22400 in response to drought stress and ABA, which occurs within 15-30 minutes. At1g22400 expression would not only assist in closure, but also prepare stomata for rapid opening by creating a readily available pool of stored cytokinins. However, this model would require opposite fluctuations in GT and *β*-glucosidase activity. However, we lack evidence of cytokinin *β*-glucosidase expression in guard cells.

Future research directions

Spatial and temporal expression patterns were determined for the Arabidopsis cytokinin glucosyltransferases (Chapter 3) and have also been established for other cytokinin biosynthetic and metabolic genes such as the Arabidopsis *IPT*s (Miyawaki et al., 2004), CKXs (Werner et al., 2003) or *β*-

glucosidases (Gu et al., 2006). However, a comprehensive study of GUS expression for all these promoter-GUS lines, combined with short-term RT-PCR analyses of the wild type, would be required to elucidate the dynamics and interactions of cytokinin gene expression. For instance, the effects on gene expression by stress and hormones could be determined, leading to an integrated model of the dynamics of cytokinin biosynthesis and metabolism under various conditions. These studies could be complemented by cytokinin analyses, which would reflect the final result of differential enzyme activities. Such study would have the potential to answer whether O-glucosyltransferases and *β*-glucosidases interact to regulate levels of active aglycones or whether O-glucosylation, together with degradative oxidation, simply serves the purpose of downregulating excessive levels of cytokinins and the plant relies on IPT-mediated *de novo* cytokinin biosynthesis to increase cytokinins when needed.

The most interesting but puzzling findings of the maize study were the increased chlorophyll and tasselseed phenotypes of transformants. If the *Ubi:ZOG1* transformants would be grown alongside maize IPT, *β*-glucosidase and CKX overexpressors, careful comparisons could be made between these lines and periodic samples could be taken throughout the life cycle for cytokinin and RT-PCR analysis. In addition to the cytokinin genes, also feminization-related genes such as tasselseed2 (DeLong et al., 1993) could be quantified. This would reveal not only long-term effects on plant development and cytokinin homeostasis, but also the genes responsible for cytokinin increase following *ZOG1* overexpression and the particular cytokinin metabolites linked to chlorophyll retention and tassel feminization in maize.

Further research may provide support for the proposed role of At1g22400 as part of a drought/ABA-induced regulatory pathway in guard cells. Studies with knock-out mutants of At1g22400 could determine whether At1g22400 expression has any effects on stomatal aperture. According to our hypothesis that cytokinin O-glucosylation plays a role in guard cell movement, inactivating the function of At1g22400 would result in slower response in stomatal closure due to the higher levels of aglycones. However, because of possibly overlapping functions of the O-

glucosyltransferases and CKXs, insertional mutants of the At1g22400 gene alone may not have a discernible effect and multiple disruptions affecting all these genes may be required. Yet, the question of whether or not O-glucosides are reactivated in stomatal control is difficult to answer because of the limitations of current tools of cytokinin analysis. Moreover, no cytokinin-specific *β*-glucosidases have been identified in Arabidopsis to date and therefore, it is not known whether any of these genes are expressed in the guard cells.

The sequencing of the Arabidopsis genome and ensuing studies of the functional genomics have led to tremendous progress in our understanding of hormone biology. The discovery of additional genes and further characterization of known genes involved in cytokinin biosynthesis, metabolism, and signaling, as exemplified by the cytokinin-activating *Lonely Guy* (*LOG*) gene (Kurakawa et al., 2007) or novel type A and B response regulators (*ARR*s) (Jain et al., 2006b; Yokoyama et al., 2007), will enhance our understanding of the mechanisms controlling cytokinin levels and the significance of the various metabolites. It is possible that additional signal transduction pathways will be uncovered or additional receptor sites besides the plasma membrane, which may provide explanations of the need for compartmentation of cytokinin biosynthesis and metabolism. Furthermore, improvements in cytokinin localization methods will be central to explaining cytokinin homeostasis at the sub-cellular level.

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