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Changes in Concentrations of Cytokinins (CKs) in Root and Axillary Bud Tissue of Miniature Rose Suggest that Local CK Biosynthesis and Zeatin-Type CKs Play Important Roles in Axillary Bud Growth

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Abstract

Involvement of cytokinins (CKs) in axillary bud growth of miniature rose was studied. Variation in root formation and axillary bud growth was induced by two indole 3-butyric acid (IBA) pretreatments in two cutting sizes. At six physiological developmental stages around the onset of axillary bud growth, concentrations of CKs were determined in both root and axillary bud tissue by liquid chromatography combined with electrospray tandem mass spectrometry (LC-ESP-MS/MS). Chronological early onset of axillary bud growth occurred in long cuttings pretreated at low IBA concentration, whereas physiological early root formation was associated with long cuttings and high IBA concentration. The CKs zeatin (Z), isopentenyl adenine (iP), zeatin riboside (ZR), dihydrozeatin riboside (DHZR), isopentenyl adenosine (iPA), zeatin *O*-glucoside (ZOG), zeatin riboside *O*-glucoside (ZROG), zeatin riboside 5′-monophosphate (ZRMP), and isopentenyl adenosine 5′-monophosphate (iPAMP) were detected. Concentrations of CKs in axillary bud tissue far exceeded those in root tissue. Indole 3-butyric acid pretreatment influenced the concentration of CKs in axillary bud tissue more than did cutting size, whereas pretreatments only slightly affected CKs in root tissue. The dominant CKs found were iPAMP and ZR. An early and large increase in iPAMP indicated rapid CK biosynthesis in rootless cuttings, suggesting that green parts, including the axillary bud, can synthesize CKs. At

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the onset of axillary bud growth an increase in concentration of Z, ZR, ZRMP, ZOG, and ZROG was largely coincident with a decrease in iPAMP, iPA, iP, and DHZR. After the onset of axillary bud growth, CK content largely decreased. These results strongly indicate a positive role for CKs in axillary bud growth, and presumably ZRMP, ZR, and Z are active in miniature rose.

Key words: Axillary bud growth; Cytokinins; Liquid chromatography-electrospray tandem mass spectrometry (LC-ESP-MS/MS); *Rosa*

INTRODUCTION

Recent reviews concerning aspects of cytokinin (CK) regulation of plant development, including axillary bud growth, noted *i.a.*: In general, shoot- and root meristems produce phytohormones, coordinate morphogenic processes via phytohormone gradients, and integrate signals from other organs (Werner and others 2001). Cytokinins regulate cell division, root and shoot growth and branching, chloroplast development, leaf senescence, stress response, pathogen resistance, and sink strength (Mok and Mok 2001; Schmülling 2002; Kakimoto 2003), and CK levels are influenced by light, stresses, and nutrition (Zazimalova and others 1999). The free bases N^6-(Δ^2-isopentenyl) adenine (iP) and zeatin (Z) are receptors proven active (see for example, Kakimoto 2003), but the *O*-glucosides and the phosphorylated ribosides can be converted back to iP and Z, whereas the 7- and 9-glucosides of Z are biologically inactive and extremely stable (Mok and Mok 2001; Mader and others 2003). The active CK pool is regulated during biosynthesis by uptake from extracellular sources, metabolic interconversions, inactivation, degradation, and signal transduction and transport (Zazimalova and others 1999; Mok and Mok 2001; Kakimoto 2003).

Evidence from hormonal studies suggests that apically produced auxin suppresses the onset of axillary bud growth, which is promoted by CKs (Tamas 1995), but it has not been demonstrated unequivocally that root-derived CKs trigger release of axillary buds (Schmülling 2002). Thus generally, axillary bud growth promotion is thought to be a consequence of endogenous auxin lowering and CK enhancing (Stafstrom 1993). Mader and others (2003) showed that at the onset of lateral bud growth in chickpea (*Cicer arietinum*) a reduction in CK catabolism coincided with a decrease in auxin, and in axillary buds zeatin riboside (ZR) dominated, whereas in mature roots the early pathway N^6-(Δ^2-isopentenyl) adenosine (iPA) form dominated.

As long as the shoot apex is intact and active in cut roses, the axillary buds remain inhibited by apical dominance. Cockshull and Horridge (1977) and Dieleman and others (1998) proposed that the auxin export from the apex decreases and the CK export from roots increases when the terminal flower bud is visible. In the rose, stem length below the node of a cutting influences root formation and axillary bud growth, and exogenous auxins advance root formation (Al-Saqri and Alderson 1996; Bredmose and others 2004). In roses, van Staden and others (1981) and van Staden (1982) found Z and ZR, Dieleman and others (1997b, 1998) quantified ZR, iP-compounds and zeatin riboside 5′-monophosphate (ZRMP), and Zieslin and Algom (2004) found Z, ZR, iP, and iPA, with different CKs dominating at different growth phases and in different organs. However, knowledge of the function of CKs in rose plants remains limited (Zieslin and Algom 2004).

The earliest onset of growth has been observed in axillary buds, which originally were positioned most distal from the root system on rose plants (Bredmose and others 1999). Thus it has been surmised (Bredmose and others 1999; Bredmose 2003) that axillary bud growth may be mediated by altered metabolism of CKs in the green parts of a rose plant, in addition to synthesis in the roots. After shoot decapitation, CK may be part of endogenous signals involved in axillary bud growth stimulation (Turnbull and others 1997). In both pea (Balla and others 2002) and chickpea (Mader and others 2003) CKs seemed to be synthesized directly in the buds, and in young rose leaves (Dieleman and others 1997b) results indicated *de novo* CK synthesis.

The objective of the present research was to determine the relationships between root formation, axillary bud growth, and endogenous levels of CKs. Previous research with the rose (Bredmose and others 2004) revealed that root formation in ultrashort cuttings was delayed only after pretreatment at low indole 3-butyric acid (IBA) concentrations; thus, IBA could substitute for cutting size. Onset of axillary bud growth was also delayed in ultrashort cuttings, and IBA at 10^{-4}–10^{-3} M was optimal for axillary bud growth. Axillary bud growth and root visibility often occurred simultaneously, indicating that preceding root formation

was not necessary for axillary bud growth (Bredmose and others 2004).

No reports detailing the concentration changes in CKs in axillary bud and root tissue before, during, and after onset of axillary bud growth in rose plantlets have been found. In the present investigation with different sized rose cuttings pretreated with different IBA concentrations, the role of endogenous CKs in rose root–shoot relations was studied: in particular, the presumed involvement of CKs in axillary bud growth. The hypothesis was that axillary bud growth is triggered by enhanced bud CKs both locally and globally derived.

Materials and Methods

Plant Material

Stock plants of *Rosa* L. "Poulra002"^{N}, Heidi, Parade-Group, spaced at approximately 36 plants m^{-2} of bench, were grown commercially at Ove Nielsen Nurseries, DK-5672 Broby, Denmark. Material for cuttings was commercially cut by machine (second cut) and stored at 3°C overnight. Single-node stem cuttings with one five-leaflet leaf were excised from shoots with a visible flower bud. Cuttings were excised from medial node positions and with an internode length of either 5 mm or 20 mm below the leaf. Cutting excision and planting were carried out on 16 January.

Plant Propagation and Growth

The cutting base was treated with IBA at 10^{-5} M or 10^{-2} M using the quick-dip method (Blazich 1988) for 1–2 s, before the cuttings were inserted into peat (Pindstrup 2, Pindstrup Mosebrug A/S, Denmark) in 5.5-cm netpots (Type 6N OS Plastic A/S, Denmark), at the Research Centre Aarslev.

The netpots were placed at approximately 100 plants m^{-2} under a white polyethylene cover on benches in a glasshouse. Average air and root temperatures during the experiment were 23.7° and 24.3°C, respectively. Natural and supplementary light provided a photosynthetic photon flux density (PPFD) level at 56 μmol m^{-2} s^{-1} (actual measured average value). Supplementary lighting was supplied by 400-W high-pressure sodium lamps (Philips SON-T 400) for 20 h day^{-1}.

Cutting and plantlet growth and CK content were measured at six physiological/morphological developmental stages: (1) untreated cutting, at planting, day 0; (2) before axillary bud swelling; (3) at the beginning of axillary bud swelling, axillary bud about 2 mm; (4) at the visibly defined onset of axillary bud growth, axillary bud = 5 mm; (5) at axillary bud elongation, shoot about 15 mm long; and (6) at axillary shoot elongation, axillary shoot 45 mm long.

Growth Measurements

Time from planting until each developmental stage was recorded, and during sampling the number of root primordia/primary roots was recorded and the length of the longest root was measured for each plant. Fresh weight (FW) of samples for analysis was recorded to determine concentration of CKs.

For CK analysis (1) axillary buds with surrounding tissue—upper 2.5 mm stem of cutting without leaf—and (2) root tissue—lower 2.5 mm stem of cutting with roots—were sampled. Analyses of axillary bud tissue were carried out at all 6 developmental stages, whereas root tissue analyses were made only for stages 2–5. Samples were placed in PP test tubes at – 80°C.

Experimental Design and Statistics

Four hundred cuttings were planted for each of the four combinations of IBA pretreatment and cutting size. These 1600 cuttings were planted in rows of 10, and the rows were randomized to either side of a greenhouse bench. When 20 cuttings had reached each developmental stage within each treatment combination, they were sampled for CK analyses. For stages 5 and 6, only plant materials from 10 plantlets were sampled, because of the increase in mass. Sampling of root and axillary bud material within each treatment combination and developmental stage was replicated twice. For axillary bud analyses, 48 samples, that is, 2 cutting sizes × 2 IBA pretreatments × 6 developmental stages × 2 replications; and for root analyses 32 samples, that is, 2 cutting sizes × 2 IBA pretreatments × 4 developmental stages × 2 replications, were collected.

Statistical analyses of each CK were made by the GLM procedure (SAS Institute 1990) with IBA pretreatment, cutting size, and developmental stage (only stages 2–5) and their interactions included in the model. $LSD_{0.99}$ values for each CK were determined from pairwise comparisons and are shown in Figures 1–3. Axillary bud tissue data for iPA, iP, and dihydrozeatin riboside (DHZR), and root tissue data for ZR, iPA, and N^6-(Δ^2-isopentenyl) adenosine 5′-monophosphate (iPAMP) were square root transformed to ensure variance homogeneity. Root data for Z, DHZR, zeatin *O*-glucoside (ZOG), and zeatin riboside *O*-glucoside (ZROG) were not statistically analyzed because the concentrations of CKs in more than 30% of the samples were below the detection limit. Thus, only mean values are

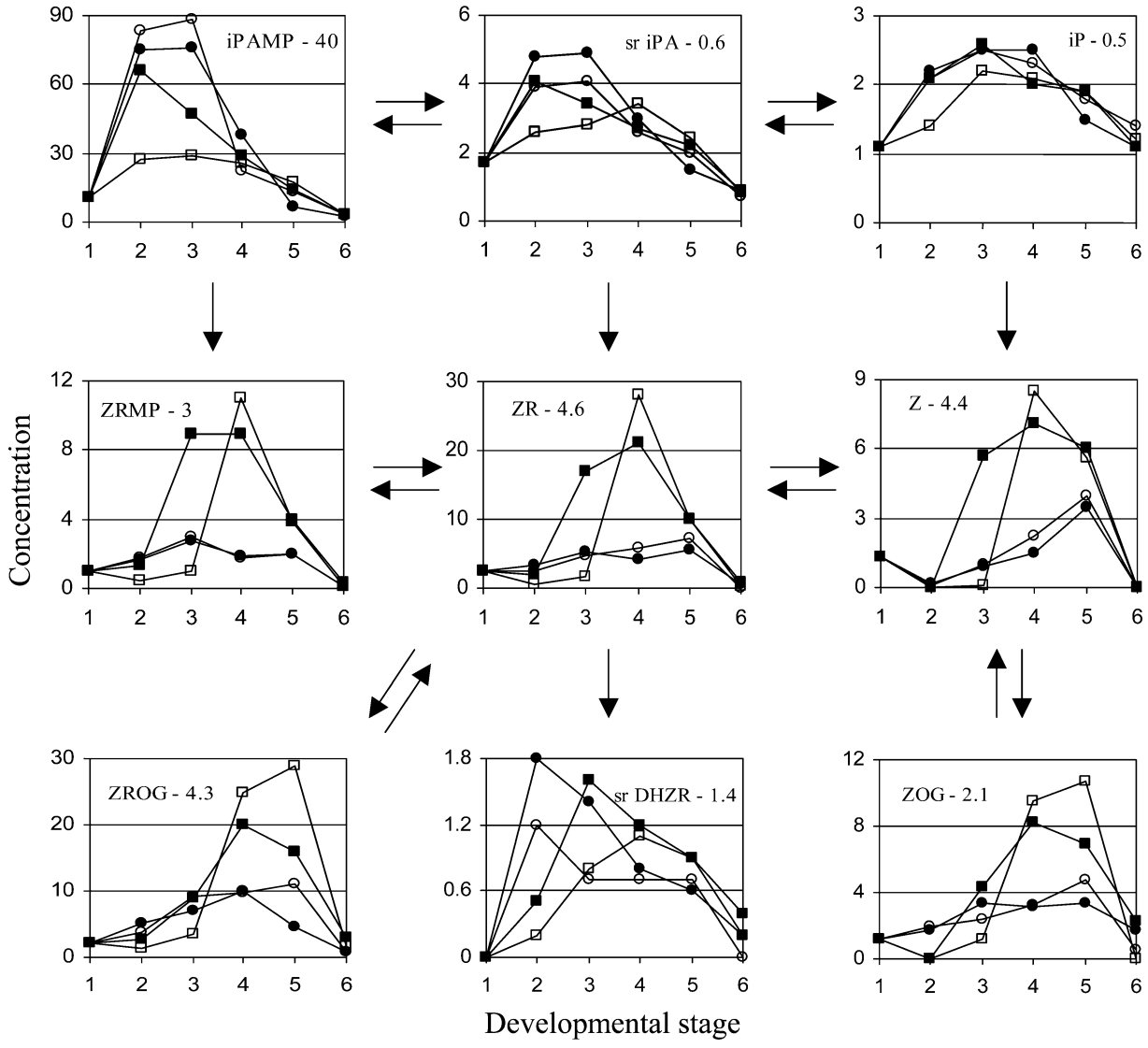

Figure 1. Concentration of CKs in axillary bud tissue at developmental stages 1–6 (1 = at planting, 4 = at onset of axillary bud growth; see text) depending on pretreatment combinations (IBA[indole 3-butyric acid]/cutting size: 10^{-5} M/5 mm; 10^{-5} M/20 mm; 10^{-2} M/5 mm; 10^{-2} M/20 mm; see text). Squares and circles are 10^{-2} M and 10^{-5} M IBA pretreatment, respectively. Open and filled symbols are 5 and 20 mm cutting size, respectively. Concentrations are pmol g^{-1} FW, except for iPA (isopentenyl adenosine), iP(isopentenyl adenine), and DHZR (dihydrozeatin riboside), where square root transformations of pmol g^{-1} FW are shown. Values inserted after name of a cytokinin are $LSD_{0.99}$ for concentration at developmental stages 2–5. Arrows indicate probable CK biosynthesis in the iPAMP-dependent pathway.

shown in Figures 1–3 for these CKs. Pearson's correlation coefficients between CKs were also determined (SAS Institute 1990).

Standard Samples

Deuterated cytokinin tracers ($^2H_5$-Z, $^2H_5$-ZR, $^2H_5$-ZOG, $^2H_5$-ZROG, $^2H_3$-DHZ, $^2H_3$-DHZR, $^2H_5$-Z9G, $^2H_5$-Z7G, $^2H_6$-iP, $^2H_6$-iPA) and unlabeled standards for Z, ZR, ZOG, ZROG, dihydrozeatin (DHZ), DHZR, zeatin 9-glucoside (Z9G), zeatin 7-glucoside (Z7G), iP, and iPA were purchased from Apex Organics (Honiton, UK). Deuterated dihydrozeatin 9-glucoside (DHZ9G) was not available.

Sample Preparation

The extraction and purification methods followed the method of van Rhijn and others (2001). In brief, frozen plant material was ground in liquid N_2, and about 500 mg of homogenized tissue from each sample was extracted in 5 ml precooled

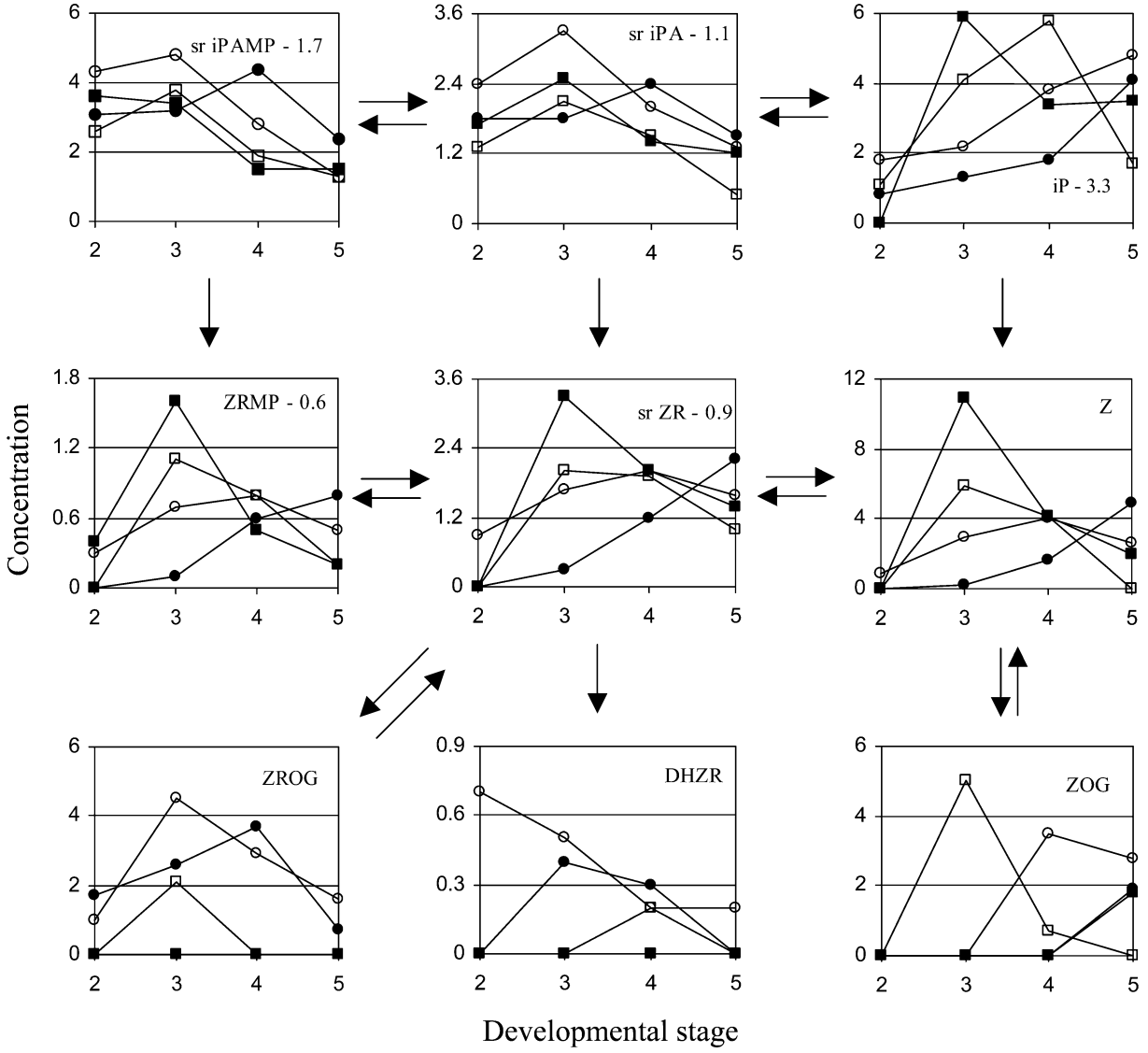

Figure 2. Concentration of CKs in root tissue at developmental stage 2 to 5 (2 = before axillary bud swelling, 4 = at onset of axillary bud growth, see text) depending on pretreatment combinations (IBA/cutting size: 10^{-5} M/5 mm; 10^{-5} M/20 mm; 10^{-2} M/5 mm; 10^{-2} M/20 mm; see text). Squares and circles are 10^{-2}- M and 10^{-5}- M IBA pretreatment, respectively. Open and filled symbols are 5 and 20 mm cutting size, respectively. Concentrations are pmol g^{-1} FW, except for iPAMP, iPA, and ZR (zeatin riboside), where square root transformations of pmol g^{-1} FW are shown. Values inserted after name of a cytokinin are $LSD_{0.99}$ for concentration at developmental stages 2–5. Arrows indicate probable CK biosynthesis in the iPAMP-dependent pathway.

CH_3OH–$CHCl_3$–HCO_2H–H_2O solution (12:5:2:1 v/v) and stable isotope-labeled CKs were added as internal standards. The samples were extracted overnight at –20°C. After centrifugation, CKs were extracted from the supernatant by using 500 mg strong cation-exchange cartridge columns (SCX) (Varian, Harbor City, CA) pre-equilibrated with CH_3OH–H_2O–HCO_2H (25:24:1, v/v). After the cartridge was washed with extraction buffer and CH_3OH, CKs were eluted with CH_3OH–4 M aqueous NH_3 (3:2 v/v) and the eluate was reduced under vacuum pressure to aqueous phase, as described by Aastot and others (1998). The aqueous samples were diluted with 10 mM ammonium formate to a volume of 4 ml and further purified on a 0.5 ml DEAE-Sephadex anion exchanger (Sigma-Aldrich, St. Louis, MO) in series with a 500 mg Bond Elut-C_{18} (Varian, Harbor City, CA). Columns were separated after washing with 10 mM ammonium formate. The nucleotides were eluted from the anion exchanger with 1 M ammonium formate, and bases, nucleosides, and glucosides

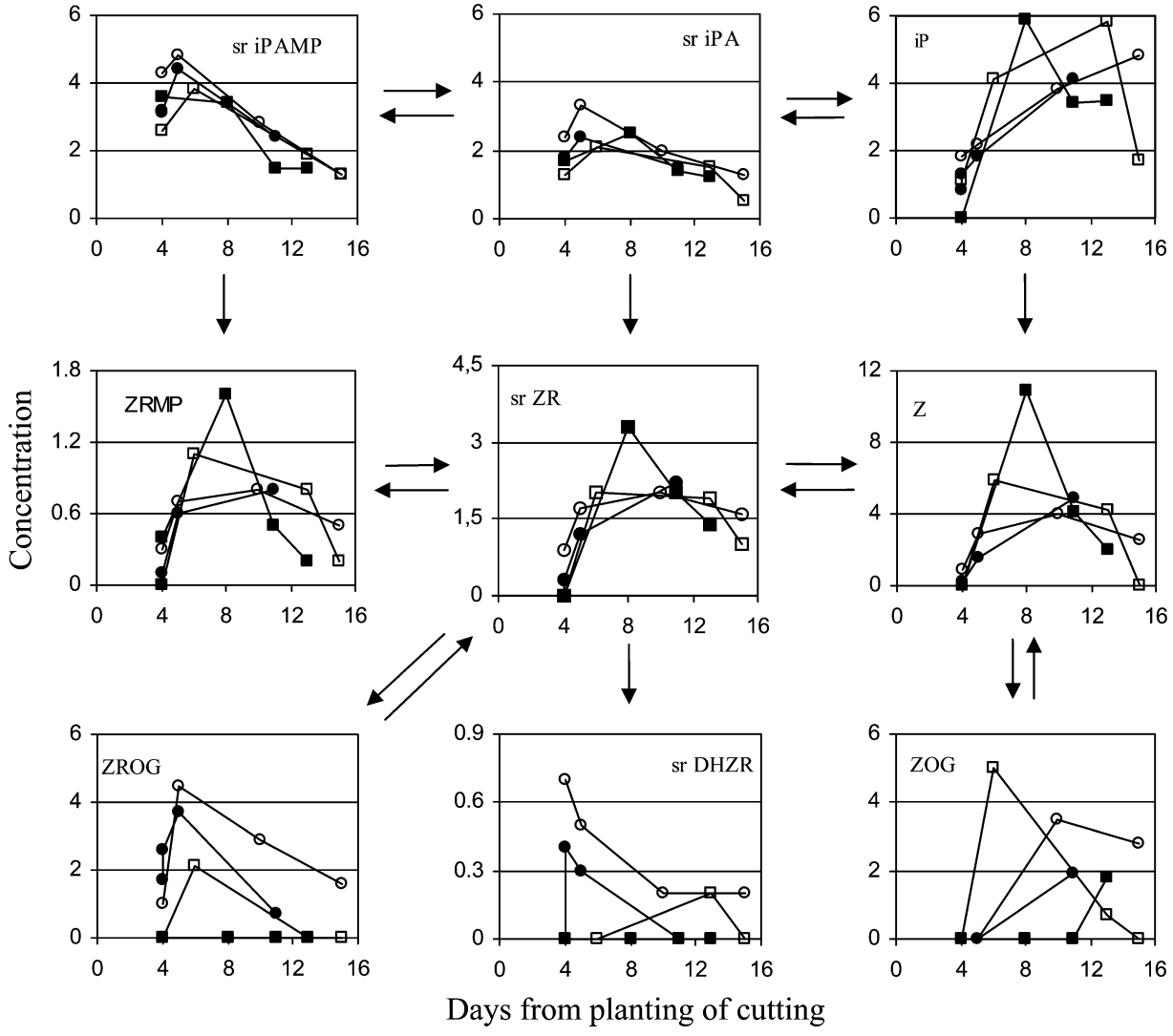

Figure 3. Concentration of CKs in root tissue as a function of time from planting of cutting depending on pretreatment combinations (IBA/cutting size: 10^{-5} M/5 mm; 10^{-5} M/20 mm; 10^{-2} M/5 mm; 10^{-2} M/20 mm; see text). Squares and circles are 10^{-2} M and 10^{-5} M IBA pretreatment, respectively. Open and filled symbols are 5 and 20 mm cutting size, respectively. Concentrations are pmol g^{-1} FW, except for iPAMP, iPA, and ZR, where square root transformations of pmol g^{-1} FW are shown. Values inserted after the name of a cytokinin are $LSD_{0.99}$ for concentration at developmental stages 2–5. Arrows indicate probable CK biosynthesis in the iPAMP-dependent pathway. *Note:* The CK data used are the same as in Figure 2.

were eluted from the C_{18} cartridge with CH_3OH–H_2O–CH_3CO_2H (80:20:1 v/v). Nucleotides were converted into nucleosides by alkaline phosphatase treatment (Alkaline type III-L Bacteri, Sigma-Aldrich, St. Louis, MO) at 37°C for 30 min and purified on a 500 mg C_{18} cartridge. The converted nucleotide samples were analyzed separately as described below. All samples were evaporated to dryness and reconstituted in 400 µl CH_3CN–H_2O–glycerol–HCO_2H (20:78:1:1 v/v) solution and analyzed by liquid chromatography–electrospray tandem- mass spectrometry (LC-ESP-MS/MS).

Liquid Chromatography–Electrospray Tandem-Mass Spectrometry

Free CK-bases, nucleosides, and glucosides were separated on a symmetry C_{18} column (3.5 µm, 2.1 × 150 mm) (Waters, Milford, MA) operated at 40°C and a flow rate of 0.4 ml min^{-1}, using a gradient between solvent A: 0.1% CH_3CO_2H, with pH adjusted to 8.0 with NH_3, and solvent B: CH_3OH. The profile used was initially 15% B; 10 min 41.5% B; 15.0 min 45% B; 15.5 min 90% B; 16.5 min 90% B; and 17.0 min 15% B. All changes were linear pro-

Table 1. Mass Transitions used in the Three Segments of the Multiple Reaction Monitoring Method (MRM) and the Corresponding Cone Voltage and Collision Energy

Name	Formula	MRM segment	$[M + H]^+$	Fragment	Cone (eV)	Collision energy (eV)	LOD (fmol)[a]
Z7G	$C_{16}O_6N_5H_{23}$	I	382	220	30	21	0.3
d_5-Z7G	$C_{16}O_6N_5H_{18}d_5$	I	387	225	30	21	
Z9G	$C_{16}O_6N_5H_{23}$	I	382	220	30	21	0.1
d_5-Z9G	$C_{16}O_6N_5H_{18}d_5$	I	387	225	30	21	
DHZ9G	$C_{16}O_6N_5H_{25}$	I	384	222	30	25	0.4
Z	$C_{10}ON_5H_{13}$	II	220	136	30	20	1.0
d_5-Z	$C_{10}ON_5H_8d_5$	II	225	136	30	20	
ZR	$C_{15}O_5N_5H_{21}$	II	352	220	30	20	0.4
d_5-ZR	$C_{15}O_5N_5H_{16}d_5$	II	357	220	30	20	
DHZ	$C_{10}ON_5H_{15}$	II	222	136	30	22	1.9
d_3-DHZ	$C_{10}ON_5H_{12}d_3$	II	225	136	30	22	
DHZR	$C_{15}O_5N_5H_{23}$	II	354	222	30	20	1.3
d_3-DHZR	$C_{15}O_5N_5H_{20}d_3$	II	357	222	30	20	
iP	$C_{10}N_5H_{13}$	III	204	136	26	19	0.9
d_6-iP	$C_{10}N_5H_7d_6$	III	210	136	27	19	
iPA	$C_{15}O_4N_5H_{21}$	III	336	204	30	20	0.7
d_6-iPA	$C_{15}O_4N_5H_{15}d_6$	III	342	210	26	20	

Z: zeatin; ZR: zeatin riboside; DHZ: dihydrozeatin; DHZR: dihydrozeatin riboside; iP: isopentenyl adenine; iPA: isopentenyl adenosine; [M + H], protonated molecular ion:
[a]The limits of detection (LOD) (S/N = 3) correspond to 2 µl injection solution.

grammed. The injection volume was 1 µl. The high performance liquid chromatography (HPLC) system from Agilent (Waldbronn, Germany) consisted of a G1399A thermostated wellplate sampler, a G 1312A binary pump, and a G1316A column compartment. The eluate was spited in a ratio of 1:1 before entering the electrospray source of the Quattro LC from Micromass (Manchester, UK). MassLynx 3.5 from Micromass was used for control of the HPLC and data processing. The settings of the electrospray were: Desolvation gas (N_2) 600 l h^{-1} at 350°C, capillary voltage 3.0 kV, and source temperature 120°C. For the multiple reactions monitoring method, argon at a pressure of 2.5 mTorr was used as the collision gas. The cone voltage as well as the collision energy was set individually for each of the CKs (Table 1). Data were collected in three segments: segment 1, from 0–7 min; segment 2, 7.5–12 min; and segment 3, 15.5–17 min.

Mass spectrometry and tandem MS spectra of the individual compounds were recorded by continuously infusing a solution containing 10 µmol l^{-1} at a speed of 10 µl min^{-1} in the flow stream just before the electrospray when eluting the column with 15% mobile phase B (200 µl min^{-1}). Identical fragmentation pattern for isotopically labeled internal standards and the corresponding unlabeled compounds were used, except for DHZ9G for which DZ9G was used as internal standard. Quantification was achieved by using deuterium-labeled analogues and applying linear regression to the response factor versus concentration data.

Results

The study focused on CKs in stem cuttings of miniature rose plants containing a single dormant bud. The variables tested were cutting length, concentration of IBA applied to the cutting base prior to propagation, and the developmental stage of the axillary buds.

Root Formation, Axillary Bud Growth, and Time

Visible onset of axillary bud growth, developmental stage 4, was recorded 5 days after planting on 20-mm cuttings pretreated with 10^{-5} M IBA (Table 2). At that time only 0.2 roots $plant^{-1}$ had been formed. In 5-mm cuttings pretreated with 10^{-2} M IBA the onset of axillary bud growth was not until 13 days after planting when 23 roots $plant^{-1}$ were present (Table 2). Plantlets from the other two combinations of cutting size and IBA pretreatment had intermediate time for onset of axillary bud growth and number of roots.

Many (20) roots were quickly formed in long cuttings pretreated with high IBA concentration—that is, 8 days after planting and at develop-

Table 2. Time (number of days) to each Physiological Developmental Stage (Ds), Number of Roots Plant^{-1} (No.), and Length of Longest Root (mm) Depending on IBA Pretreatment (IBA) and Cutting Size (CS) at Physiological Developmental Stages 2–5

	Developmental stage[a]											
	2			3			4			5		
	Ds	No.	mm	Ds	No.	mm	Ds	No.	mm	Ds	No.	mm
IBA/CS												
10^{-5} M/5 mm	4	0	0	5	0.4	0.1	10	8	16	15	12	46
10^{-5} M/20 mm	4	0.1	0	4	0.1	0	5	0.2	0	11	12	30
10^{-2} M/5 mm	4	0.1	0	6	2.9	0.3	13	23	42	15	24	45
10^{-2} M/20 mm	4	0.1	0	8	20	13	11	22	36	13	25	39

IBA: indole 3-butyric acid.
[a]*Stage 4 is onset of visible axillary bud growth.*

mental stage 3—whereas, after low IBA pretreatment, fewer roots (12) were formed more slowly (11 days after planting) and later (at stage 5) (Table 2). The earliest formation, at stage 3, of a long root (13 mm), was from long cuttings pretreated with high IBA concentration (Table 2). At developmental stage 4, the longest roots, 36–42 mm, were observed in cuttings pretreated with high IBA concentration, whereas at that stage the longest roots in cuttings pretreated at low IBA concentration measured only 0–16 mm (Table 2). Pretreatment at 10^{-2} M IBA increased root number and rate of root formation compared with 10^{-5} M IBA, whereas 20-mm cuttings hastened axillary bud growth compared with 5-mm cuttings (Table 2).

CKs in Rose Tissues

The CKs, Z and iP (free bases); ZR, iPA, and DHZR (ribosides); ZOG and ZROG (glucosylated); ZRMP and iPAMP (nucleotides) were detected in both axillary bud and root tissue. Even though the limits of detection for the LC-ESP-MS/MS method ranged from 0.1 to 1.9 amol g^{-1} FW, similar to the limits reported by van Rhijn and others (2001), it was not possible to detect DHZ, Z9G, Z7G, or DHZ9G (Table 1).

The CKs consistently found in large amounts in both tissues were iPAMP, ZR, ZROG, iPA, ZRMP, and Z (Figures 1, 2). The CK levels were the highest in the axillary bud tissue, and in both tissues, iPAMP far exceeded the concentrations of other CKs (Figures 1, 2).

CKs in Axillary Bud Tissue

The most abundant CK in the axillary bud tissue was iPAMP, with concentrations up to about 30–90 pmol g^{-1} FW depending on treatment, having increased 3–9-fold from developmental stage 1 (Figure 1). The CKs ZROG, ZR, and iPA were found with concentrations up to 24–29 pmol g^{-1} FW, whereas ZRMP, ZOG, Z, and iP were found in concentrations of 7–11 pmol g^{-1} FW. Dihydrozeatin riboside was present only at low concentrations corresponding to 0.8–3.4 pmol g^{-1} FW (Figure 1). The relative CK profile found in the axillary buds on average was iPAMP > iPA > ZROG > ZR > iP > ZOG > ZRMP > Z > DHZR.

At the onset of axillary bud growth (defined as stage 4), the concentrations of ZRMP, ZR, and Z in axillary buds were at their highest, whereas iPAMP, iPA, DHZR, and iP were highest at earlier stages (Figure 1). For CKs early in the biosynthetic pathway (iPAMP and iPA) the concentration was higher in cuttings pretreated at low IBA concentration, whereas for CKs later in the pathway (ZR, Z, ZRMP, ZROG, and ZOG), the concentration was higher in cuttings pretreated at the high IBA concentration (Figure 1).

The concentration of all nine CKs detected in axillary bud tissue varied significantly with developmental stage, IBA pretreatment, and cutting size, with a larger effect of IBA pretreatment than of cutting size (Table 3). Three-way interactions between the experimental treatments were significant for iPAMP, iPA, ZR, ZRMP, and ZROG ($p < 0.004$) (Table 3). Further, interactions between developmental stage and IBA pretreatment were found for iP ($p = 0.003$), Z ($p = 0.008$), DHZR ($p = 0.01$), and ZOG ($p < 0.0001$) (Table 3). Irrespective of cutting size, iPAMP peaked in plantlets pretreated with low IBA at stages 2–3 at concentrations above 75 pmol g^{-1} FW (Figure 1). High IBA pretreated plantlets from large cuttings showed a similar response, whereas in small cuttings, iPAMP did not exceed 30

Table 3. Analysis of Variance (p-values) for each Phytohormone in Axillary Bud and Root Samples including Second- and Third-order Interactions

	sr-IPAMP	sr-iPA	iP	sr-ZR	Z	ZRMP	sr-DHZR	ZROG	ZOG
Axillary bud									
Stage (S)	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.5	0.0001	0.0001
Auxin (A)	0.0001	0.0001	0.03	0.0001	0.0003	0.0001	0.7	0.0001	0.0001
Cutting (C)	0.03	0.0001	0.03	0.1	0.5	0.05	0.07	0.0005	0.2
S x A	0.0001	0.0001	0.003	0.0001	0.008	0.0001	0.01	0.0001	0.0001
S x C	0.08	0.0001	0.02	0.0001	0.1	0.002	0.3	0.0001	0.0001
A x C	0.006	0.3	0.02	0.03	0.2	0.04	0.9	0.4	0.5
S x A x C	0.004	0.003	0.04	0.0001	0.2	0.001	0.9	0.001	0.03
	sr-IPAMP	sr-iPA	iP	sr-ZR	Z	ZRMP	sr-DHZR	ZROG	ZOG
Root									
Stage (S)	0.0001	0.0001	0.0003	0.0001	–	0.0003	–	–	–
Auxin (A)	0.0009	0.001	0.2	0.08	–	0.3	–	–	–
Cutting (C)	0.9	0.9	0.2	0.4	–	0.9	–	–	–
S × A	0.06	0.5	0.001	0.0001	–	0.0006	–	–	–
S × C	0.04	0.1	0.08	0.004	–	0.5	–	–	–
A × C	0.6	0.02	0.1	0.0002	–	0.1	–	–	–
S × A × C	0.01	0.05	0.4	0.004	–	0.07	–	–	–

iPAMP: isopentenyl adenosine 5′-monophosphate; iP: ; iPA: ; ZR: zeatin riboside; ZRMP: zeatin riboside 5′-monophosphate; ZROG: zeatin riboside O-glucoside; ZOG: zeatin-O-glucoside.

Note: Data for phytohormones preceded by sr have been square root transformed prior to analysis to ensure variance homogeneity. The concentration of ZROG, ZOG, Z, and DHZR in root tissue was, for many samples, below the detection limit; thus statistical analyses were not possible (–).

pmol g^{-1} FW (Figure 1). Isopentenyl adenine iPA responded similarly (Figure 1); iP peaked at stage 3–4 with only small effects of IBA pretreatment and cutting size (Figure 1). Zeatin, ZR, and ZRMP peaked in high IBA pretreated plantlets at stage 3–4 followed by a sharp decline with plantlets from short cuttings peaking later than plantlets from large cuttings (Figure 1). Zeatin, ZR, and ZRMP in low IBA pretreated plantlets were fairly constant at stages 2–5. In high IBA pretreated plantlets ZOG and ZROG peaked at stage 4–5 at approximately 10 and 25 pmol g^{-1} FW, respectively, followed by a sharp decline. For low IBA pretreated plantlets, a similar response pattern was found, but with a much smaller influence of the developmental stage (Figure 1). Dihydrozeatin riboside peaked at stage 2 or 3, depending on IBA pretreatment (Figure 1). It is possible that at developmental stage 6 much stem tissue may have had an attenuating influence on the CK concentration.

Correlations between CKs in Axillary Bud Tissue and Root Tissue

In axillary bud tissue, the concentration of iPA and iPAMP was strongly positively correlated, and these two CKs were negatively correlated with Z (Table 4). Zeatin, ZR, ZRMP, ZOG, and ZROG were all very strongly positively correlated to each other, with coefficients of correlation between 0.66 and 0.96 (Table 4). Further, relatively high correlations (0.49–0.50) between DHZR, iPA, and iP were found (Table 4). Zeatin riboside 5′-monophosphate was not significantly correlated with iPAMP (Table 4).

In root tissue, iP was significantly correlated with Z, ZR, and ZRMP, with coefficients above 0.7 (Table 4). Moreover, the pattern of correlations in root tissue was fairly similar to that in axillary bud tissue; however it was not as clear and was associated with lower coefficients (Table 4).

Coefficients of correlation between the concentration in axillary bud tissue and root tissue of each CK were insignificant (R^2 numerical below 0.42), except for iPAMP ($R^2 = 0.63$) and ZR ($R^2 = 0.49$).

CKs in Root Tissue

In general, the concentration of CKs in root tissue showed the same pattern as for axillary bud tissue; however, it was less clear and more fluctuating (Figure 2). The most abundantly found CK in root tissue was iPAMP, with highest concentrations from about 14 to 24 pmol g^{-1} FW (Figure 2). Zeatin, ZR, and iPA peaked at about 10–12 pmol g^{-1} FW, whereas

Table 4. Pearson's Coefficients of Correlations between the Phytohormones in Both Axillary Bud Tissue (BT) and Root Tissue (RT)[a]

BT	IPAMP							
iPA	0.89	iPA						
iP	0.57	0.57	iP					
Z	−0.52	−0.43	−0.17	Z				
ZR	−0.28	−0.15	0.07	0.88	ZR			
ZRMP	−0.13	−0.06	0.16	0.83	0.96	ZRMP		
ZOG	−0.46	−0.33	−0.08	0.84	0.78	0.69	ZOG	
ZROG	−0.40	−0.30	−0.07	0.81	0.75	0.66	0.95	ZROG
DHZR	0.34	0.49	0.50	−0.09	0.14	0.17	0.09	−0.06
RT	iPAMP							
iPA	0.91	iPA						
IP	−0.20	0.07	iP					
Z	0.04	0.25	0.71	Z				
ZR	0.01	0.27	0.78	0.78	ZR			
ZRMP	0.25	0.41	0.71	0.83	0.90	ZRMP		
ZOG	−0.08	−0.04	0.38	0.57	0.24	0.33	ZOG	
ZROG	0.65	0.64	−0.08	0.10	−0.11	0.11	0.21	ZROG
DHZR	0.46	0.42	−0.13	−0.14	−0.20	−0.06	−0.14	0.45

[a]*Coefficients numerical above 0.44 are significant at the 99% level.*

iP, ZOG, and ZROG did not exceed 6 pmol g^{-1} FW. Dihydrozeatin riboside and ZRMP were found only at concentrations below 1.6 pmol g^{-1} FW (Figure 2). The relative CK profile found in the roots on average was iPAMP > iPA > iP = ZR > Z > ZOG = ZROG > ZRMP > DHZR.

Three-way interactions between developmental stage, IBA pretreatment, and cutting size were significant for iPAMP and ZR (Table 3). Zeatin riboside in plantlets from short cuttings was highest at stages 3–4, independent of IBA pretreatment. Zeatin riboside in long cuttings pretreated at 10^{-2} M IBA peaked at a higher level at stage 3, whereas after pretreatment at 10^{-5} M IBA, ZR gradually increased with increasing developmental stage (Figure 2). Further, interactions between developmental stage and IBA pretreatment were significant for iP ($p = 0.001$) and ZRMP ($P = 0.0006$) (Table 3). Low IBA pretreated plantlets gradually increased in iP and ZRMP from stage 2 to stage 5, whereas high IBA pretreated plantlets peaked at stages 3–4 (Figure 2). The concentration of iPA was influenced by both developmental stage ($p < 0.0001$) and IBA pretreatment ($p = 0.001$) (Table 3), being highest (about 10 pmol g^{-1} FW) at stage 3 and after low IBA pretreatment (Figure 2).

Cytokinins in root tissue depending on time from planting of cutting (Figure 3) showed that root CKs were only slightly affected by IBA pretreatment and cutting size and were less influenced by the developmental stage *per se* and more by the time elapsed after planting (Figure 3). iPAMP peaked within the first 5 days after planting and the highest iPA concentration was found approximately 1 week after excision (Figure 3). Z, iP, ZR, and ZRMP peaked about 8–10 days after cutting excision (Figure 3). CKs in root tissue depending on the number of roots formed (data not shown) revealed that largely, only concentration of iP increased with an increase in root number (until about 20 roots $plant^{-1}$). In contrast, in general, iPAMP concentration was highest prior to root appearance, demonstrating very early biosynthesis of iPAMP in cuttings before any root was visible.

Discussion

Variation in root formation and axillary bud growth was induced in miniature rose 'Heidi' by two IBA pretreatments combined with two cutting sizes. The responses (Table 2) were in accordance with previous results (Bredmose and others 2004). Cytokinin concentrations were quantified at six physiological developmental stages around the onset of axillary bud growth in different tissues. The dominant CKs were iPAMP, iPA, ZR, and ZROG, and these differed in level depending on developmental stages, time, experimental treatment, and between axillary bud tissue and root tissue (Table 3, Figures 1, 2, 3).

The results strongly suggest that endogenous cytokinins are controlling axillary bud growth in rose (Figure 1). This is in agreement with findings in other plants (Tamas 1995), and results with cut-

roses (Dieleman and others 1998; Zieslin and Algom 2004) might also point to such a role. The greatest change was an early large increase in iPAMP in both bud- and root tissue prior to axillary bud growth (Figures 1, 2), indicating rapid CK biosynthesis directly after cutting excision. Because the proportion of the existing stem was high in the sample at early stages when the bud was small and roots were minimal and decreased once growth commenced, it was difficult to distinguish true bud/root CK from that present in adjoining stem. Because plants in early stages were mostly rootless (Table 2) and the CK concentration right after cutting excision was low (Figure 1), the present results strongly imply that the stem, leaf, and/or the axillary bud can synthesize CKs. *De novo* synthesis of CKs in green plant parts has also been reported in other plants (Balla and others 2002; Mader and others 2003; Nordström and others 2004) and results of Dieleman and others (1997b) indicate that young rose leaves might be able to synthesize CKs. Moreover, a need for local CK biosynthesis before onset of axillary bud growth has been suggested (Schmülling 2002). Isopentenyl adenosine 5′-monophosphate is regarded as the precursor of iPA and ZRMP, the latter thought to have strong promotive effects on cell division and lateral bud growth (Mader and others 2003). However, lack of correlation between the concentration of iPAMP and ZRMP (Table 4) indicates that the conversion of iPAMP to ZRMP could be of less importance in rose, or that a rapid dephosphorylation of ZRMP to ZR took place (as indicated by the negative correlation between iPAMP and Z; Table 4).

High IBA pretreated cuttings formed many roots, but the onset of axillary bud growth was delayed (Table 2). Early biosynthesis of iP-type CKs seemed suppressed in high IBA pretreated short cuttings compared to those with low IBA pretreatment, whereas high IBA pretreatment enhanced Z-type CKs at stages 3–4 (Figure 1). Indole 3-butyric acid application to cuttings has previously been reported to inhibit axillary bud growth (Sun and Bassuk 1993). Seemingly, auxin can mediate a control of the CK pool, by reducing the rate of CK biosynthesis (Nordström and others 2004); moreover, reports of IPT gene isolation in *Arabidobsis* show that CK downregulates four genes, whereas auxin upregulates two, indicating a possible auxin regulation of CK biosynthesis (Miyawaki and others 2004; Takei and others 2004). Dieleman and others (1997a, 1997b) determined the CK content in shoot and roots at three stages of rose stem development (the earliest being about the present stage 5), without being able to correlate CK import and plant development. At their stage 1, they found ZR, ZRMP, iPA, and iPAMP, whereas, at about the corresponding stage, we found those CKs, and in addition found Z, iP, DHZR, ZOG, and ZROG (Figures 1, 2). Furthermore at the stage of visible axillary bud growth, all CKs were found at higher concentrations. Van Staden (1982) demonstrated that, after decapitation of rose plants, CKs moved from the subtending leaf to the axillary bud via the phloem. In intact rose plants, a high ZR concentration in bleeding sap (root derived) appeared to coincide with axillary bud growth (Dieleman and others 1998). The present investigation showed an increase in Z, ZR, and ZRMP at axillary bud growth and a decrease afterwards (Figure 1).

At the axillary bud growth (stage 4), iPAMP, iPA, iP, and DHZR in axillary bud tissue largely declined, whereas ZR, ZROG, ZRMP, ZOG, and Z largely peaked or increased (Figure 1). These changes do not correlate directly with commencement of meristematic activity within the bud, as this occurs well before stage 3. Also, iPAMP, and iPA were negatively correlated with Z (Table 4), possibly indicating conversion. Because Z, ZR, and ZRMP are considered active CK forms, the present results may indicate that the tissues analyzed at the onset of axillary bud growth had CK profiles, like those for sink regions (Mader and others 2003). The decline in iPAMP possibly reflected an increase in its hydroxylation to ZRMP, which is then dephosphorylated to ZR. Physiologically, iP and Z have been proven to be active forms (for example, Kakimoto 2003), as receptors to recognize them have been found; ZR and ZRMP are also considered active, whereas ZOG and ZROG are considered storage forms (Mok and Mok 2001). In rose, axillary bud growth might be mediated by altered metabolism of CKs in the green plant parts, in addition to biosynthesis in the roots, as has been surmised (Bredmose and others 1999; Bredmose 2003). Moreover, that root derived CKs trigger release of axillary buds has not been demonstrated unequivocally (Schmülling 2002). In general, the results could indicate that the biosynthesis of CK was stimulated in the axillary bud tissue (sink region with active Z-type CKs), whereas leaf, stem, and root tissues (prior to that) were enhanced in early pathway isopentenyl-CKs (source regions). Further, it could be possible that ZRMP, ZR, and its immediate metabolite Z are the active CKs influencing the onset of axillary rose bud growth (Figure 1). These three CKs were very strongly positively correlated with each other (Table 4). Results of Dieleman and others (1997b) suggest that ZR in the xylem might be involved in the onset of axillary bud growth; however, no clear relationship between CK import and rose plant

development could be established (Dieleman and others 1997a). Results of Zieslin and Algom (2004) suggest a major role of iP and iPA in development and growth of shoots of fully developed 3-year-old rose plants, and differences in plant type, age, root mass, and growth may influence content of CKs. However, the present results (Figure 1) clearly indicate that, at the onset of axillary bud growth, biosynthesis of Z, ZR, and ZRMP increased. Further, in axillary buds, elevated CK levels may stimulate auxin export (Bangerth and others 2000), thereby initiating greater CK biosynthesis *in situ* (Mader and others 2003). Experiments with the rose performed in our laboratories indicated that IAA in axillary bud tissue declined at about the onset of axillary bud growth (data not published).

In the rose, in addition to CKs, regulatory roles of abscisic acid, and/or an inhibitory complex have been reported for basal axillary bud growth (Zieslin and Khayat 1983; Le Bris and others 1999). A balance between CKs and an abscisic acid/inhibitory complex is also possible, as are interactions with other growth regulators such as ethylene (Sun and Bassuk 1993). However, inhibited axillary rose buds can be released by exogenous application of CK (Ohkawa 1984), and CKs in *in vitro* culture medium are necessary for the axillary bud to complete its developmental program (Marcelis-van Acker and Scholten 1995).

Thus, in the rose, an increase in axillary bud CKs may have resulted from a combination of biosynthesis in the bud and selective loading of cytokinin ribosides into the xylem from new root primordia or roots, as reported for chickpea (Mader and others 2003). It has been proposed that CKs may be involved in mediating nitrogen-dependent root and shoot development (see for example, Schmülling 2002). It is thus possible that axillary bud growth in rose relies on increased bud CKs as a combined result of changed translocation, local biosynthesis in cutting tissues (including the axillary bud), and metabolism; co-ordinated by phytohormone interactions (such as CKs and IAA) (Mader and others 2003).

In conclusion, the results of the present investigation suggest a pivotal role of endogenous CKs in growth of axillary rose buds, and that the axillary buds and/or their surroundings must themselves be able to synthesize CKs. The main CKs in plantlets of *Rosa* "Heidi" were iPAMP and ZR. At the onset of axillary bud growth, an increase in CK biosynthesis of Z, ZR, ZRMP, and ZOG and ZROG was largely coincident with a decrease in iPAMP, iPA, iP, and DHZR. The results thus support that axillary bud growth in rose could be controlled through changes in endogenous levels of CKs, and the results might indicate that Z, ZR, and ZRMP are the active CKs. It seems plausible that the axillary bud growth was triggered by enhanced CKs from both local and global sources, (possibly mediated by IAA), which would favor the introductory hypothesis.

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