The Forms and Sources of Cytokinins in Developing White Lupine Seeds and Fruits[1]

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A comprehensive range of cytokinins (CK) was identified and quantified by gas chromatography-mass spectrometry in tissues of and in xylem and phloem serving developing white lupine (*Lupinus albus*) fruits. Analyses were initiated at anthesis and included stages of podset, embryogenesis, and seed filling up to physiological maturation 77 d post anthesis (DPA). In the first 10 DPA, fertilized ovaries destined to set pods accumulated CK. The proportion of cis-CK:trans-CK isomers was initially 10:1 but declined to less than 1:1. In ovaries destined to abort, the ratio of cis-isomers to trans-isomers remained high. During early podset, accumulation of CK (30–40 pmol ovary^{-1}) was accounted for by xylem and phloem translocation, both containing more than 90% cis-isomers. During embryogenesis and early seed filling (40–46 DPA), translocation accounted for 1% to 14% of the increases of CK in endosperm (20 nmol fruit^{-1}) and seed coat (15 nmol fruit^{-1}), indicating synthesis in situ. High CK concentrations in seeds (0.6 μmol g^{-1} fresh weight) were transient, declining rapidly to less than 1% of maximum levels by physiological maturity. These data pose new questions about the localization and timing of CK synthesis, the significance of translocation, and the role(s) of CK forms in reproductive development.

Despite substantial literature concerning the occurrence, form, and significance of cytokinins (CK) in plant development, metabolic pathways and sites of their synthesis are yet to be clearly established. There has been no unequivocal demonstration of a plant isopentenyl transferase or of its encoding gene (*ipt*) in plant tissues (Prinsen et al., 1997). Even though a number of enzymes involved in the interconversion of CK forms have been identified (Mok and Martin, 1994) the apparent lack of a mechanism to add the isopentenyl side chain to the purine ring remains as a fundamental flaw in our understanding. A number of bacteria express the *ipt* gene, and the possibility that CK are not formed by plants themselves but rather by bacterial symbionts that colonize plant tissues (Holland, 1997) has been suggested. Although there is evidence that *Rhizobium* contributes to the complement of CK in nodulated legumes (Upadhyaya et al., 1991), there has been no demonstration of more widespread plant-microbe interactions that might be described as "CK-based symbioses." Equally perplexing is a lack of clear definition of which organs are the principal sites of synthesis of CK and whether these serve as sources connected to sinks for CK by the translocation channels of xylem and phloem. Defining organ or tissue locations where the majority of CK are formed within plants would provide much needed direction in the search for CK biosynthesis proteins and their encoding genes, as well as providing a framework to assess the potential significance of any CK-synthesizing microbial symbionts. Finally, recent evidence for the predominance of what are generally regarded as inactive cis-isomers of CK in or adjacent to tissues where there is intensive cell division has further added to the uncertainty (Emery et al., 1998a). Thus, long-held views about which forms of CK are bioactive and whether the same spectrum of CK is active in all plants need to be reassessed.

Letham (1994) has summarized evidence that supports roots as a site of CK biosynthesis. However, roots are very unlikely to be the sole source of CK, and developing seeds, cambial tissues, and the shoot apex have also been implicated (Letham, 1994). In developing lupine seeds CK levels are extremely high compared with those in vegetative tissues (Davey and van Staden, 1979), and in cereal grains it is not uncommon for as much as a 500-fold transient increase in CK to occur in the endosperm for just a few days following anthesis (Brenner and Cheikh, 1995; Morris, 1997). Furthermore, exogenous application of CK to developing flowers prevents their abortion and permits the initiation of seed development (Atkins and Pigeaire, 1993). Accumulation of CK in developing seeds coincides with the highest rates of cell division (Morris, 1997), which is consistent with the idea that CK increase the sink strength of seeds for assimilates.

Whether CK are actively synthesized in developing seeds or are accumulated primarily as a result of translocation is not clear (Morris, 1997). If transloca-

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tion is a major contributor, then whether xylem or phloem is the main source also becomes a relevant question. Calculations based on estimates for the water economy of developing lupine fruits indicated that the accumulated CK could not be entirely furnished by the CK content of xylem leaving the root system (Zhang and Letham, 1990). However, developing legume fruits are principally phloem fed (Pate et al., 1977), and phloem exudates collected from wounds in the stems, racemes, or fruits of lupine indicate the presence of CK (Davey and van Staden, 1978; Summons et al., 1981; Taylor et al., 1990). Even though there is some evidence that wounding might elevate the levels of CK in phloem exudates of lupine (Atkins, 1999), potentially both translocation channels could deliver CK to the fruit.

In the present study a comprehensive range of CK was identified and quantified by gas chromatography-mass spectrometry (GC-MS) in all component tissues of the pod and seed and in xylem and phloem exudates collected at regular intervals throughout development of fruits of white lupine (*Lupinus albus*) following anthesis. Peak rates of CK accumulation were determined, and maximum fluxes from the transport fluids to the fruits were calculated based on the concentrations of CK in xylem and phloem and on the delivery of water in each channel. In lupine as in many other grain legumes, the majority of flowers abort and abscise within 10 DPA, consequently limiting podset (Pigeaire et al., 1992). Their abortion is prevented by application of CK to flowers at or just prior to anthesis (Atkins and Pigeaire, 1993), and it was of interest to document and compare changes in CK content and composition in ovaries that were destined to set with those destined to abort.

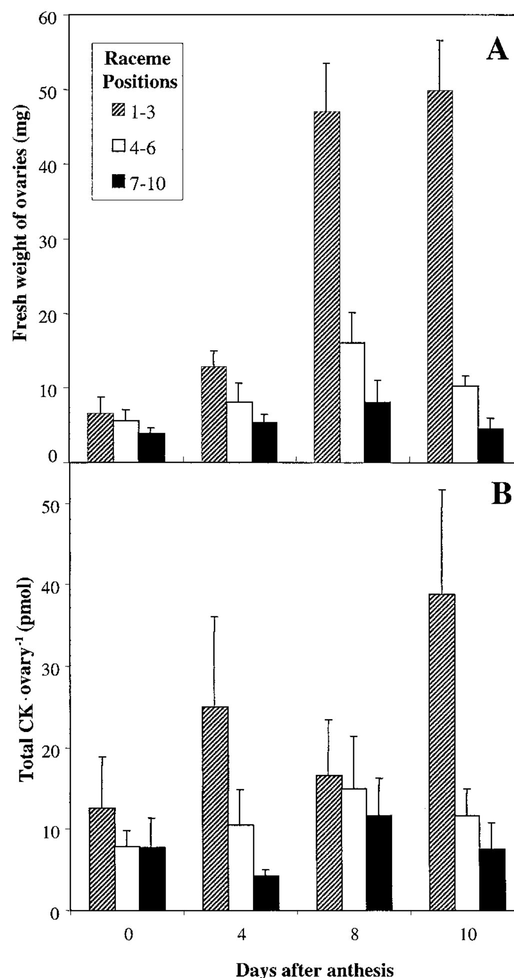

Figure 1. Increase in fresh weight (A) and change in total CK content (B) of ovaries at three different floret zones with varying probability of podset (see Fig. 3) on the mainstem raceme of white lupine. Total CK were the sum of each form shown in Table I expressed on a per ovary basis. Measurements started at anthesis and were made at intervals up to 10 DPA at each zone. Data are means ± SE ($n = 3$).

RESULTS

Effect of Floret Position on Growth and CK Composition of Developing Fruits (Ovaries) up to 10 DPA

The increases in fresh weight of ovaries at the lowest floret positions on the raceme were much greater than the average at the next group of floret positions, and at the uppermost there was essentially no growth (Fig. 1A). The ovaries at the lowest floret positions on the raceme accumulated significant amounts of CK with time (Fig. 1B). Those at intermediate positions showed a small increase following anthesis, but at the uppermost positions there was no significant accumulation of CK over the 10-d period. There were, however, significant changes in the forms of CK present in ovaries at the different positions with time (Table I).

Within each of the three zones on the raceme at anthesis ovaries contained a similar concentration of total CK with a similar composition of individual forms (Table I). In each case the nucleotides were predominant, especially [9R-MP]dihydro-zeatin ([9R-MP]DHZ) and cis-zeatin (Z) nucleotide ([9R-MP]Z), and among the ribosides, the cis-isomer of Z riboside ([9R]Z). Although the proportional concentration as cis-isomers was similar at each position at anthesis, after 4 d the proportion as cis-isomers in ovaries at the two upper zones increased significantly to around 80% of total CK recovered, but at positions 1 to 3 this did not occur (Table I). The proportion as cis-isomers subsequently declined at the upper zones and at all positions remained at 50% to 60%. During this initial 10-d period there were significant changes in concentration among the free bases and ribosides in ovaries from each zone. As a consequence the ratio of cis- to trans-isomers changed progressively from more than 10:1 to less than 1:1 by 10 DPA at positions 1 to 3, but in ovaries at the intermediate positions (4–6) it declined only to around 3:1, and at the uppermost positions (7–10) it did not decline but remained at around 10:1. Several *O*-glucosides (OG) were detected in these young

Table I. *CK composition of developing fruits (ovaries) at three different floret zones (see Fig. 3) with varying probability of podset on the mainstem raceme of white lupine*

Data are means ± SE ($n = 3$). No endogenous iP or (cis)(OG)Z was observed at any of the sampling times or floret zones, although there were good recoveries of corresponding ^{2}H-internal standards.

CK	DPA			
	0	4	8	10
	pmol cytokinin g fresh wt^{-1}			
Positions 1 to 3 on the raceme				
(cis)Z	nd[a]	98 ± 98	nd	nd
(trans)Z	15 ± 15	21 ± 21	16 ± 2	7 ± 4
DHZ	20 ± 15	30 ± 15	9 ± 5	48 ± 2
(cis)[9R]Z	453 ± 115	417 ± 89	174 ± 64	247 ± 96
(trans)[9R]Z	15 ± 4	12 ± 8	9 ± 3	48 ± 27
[9R]DHZ	8 ± 8	22 ± 11	22 ± 8	155 ± 18
[9R]iP	nd	nd	nd	nd
(cis)[9R-MP]Z	235 ± 117	187 ± 107	32 ± 22	159 ± 99
(trans)[9R-MP]Z	160 ± 80	nd	nd	nd
[9R-MP]DHZ	992 ± 604	1,166 ± 614	92 ± 43	114 ± 14
[9R-MP]iP	nd	nd	nd	nd
(trans)(OG)Z	142	22	3	11
(OG)DHZ	nd	nd	117	nd
(cis)(OG)[9R]Z	104	94	80	82
(trans)(OG)[9R]Z	nd	5	nd	4
(OG)[9R]DHZ	nd	nd	nd	nd
Total	2,144 ± 959	2,075 ± 867	553 ± 146	876 ± 260
% cis-CK	37.0	38.3	51.6	55.8
Positions 4 to 6 on the raceme				
(cis)Z	42 ± 42	24 ± 24	nd	nd
(trans)Z	3 ± 3	19 ± 19	24 ± 16	45 ± 31
DHZ	2 ± 2	16 ± 14	16 ± 12	15 ± 12
(cis)[9R]Z	368 ± 51	493 ± 121	419 ± 155	477 ± 135
(trans)[9R]Z	7 ± 3	30 ± 10	17 ± 6	34 ± 12
[9R]DHZ	13 ± 13	25 ± 14	42 ± 27	59 ± 12
[9R]iP	11 ± 11	3 ± 3	48 ± 48	nd
(cis)[9R-MP]Z	400 ± 160	387 ± 183	166 ± 99	112 ± 58
(trans)[9R-MP]Z	13 ± 10	nd	nd	nd
[9R-MP]DHZ	533 ± 88	304 ± 187	167 ± 1	343 ± 10
[9R-MP]iP	nd	nd	35 ± 35	47 ± 47
(trans)(OG)Z	nd	nd	6	nd
(OG)DHZ	nd	53	28	24
(cis)(OG)[9R]Z	nd	233	55	129
(trans)(OG)[9R]Z	nd	nd	nd	5
(OG)[9R]DHZ	nd	4	10	49
Total	1,390 ± 342	1,591 ± 551	1,032 ± 399	1,338 ± 317
% cis-CK	58.2	71.4	61.9	53.7
Positions 7 to 10 on the raceme				
(cis)Z	nd	nd	nd	nd
(trans)Z	37 ± 37	8 ± 8	nd	nd
DHZ	nd	30 ± 15	1 ± 1	71 ± 48
(cis)[9R]Z	454 ± 12	488 ± 21	563 ± 129	719 ± 271
(trans)[9R]Z	6 ± 6	10 ± 10	14 ± 8	nd
[9R]DHZ	20 ± 20	30 ± 15	14 ± 10	17 ± 8
[9R]iP	18 ± 18	nd	nd	nd
(cis)[9R-MP]Z	241 ± 58	187 ± 55	235 ± 39	94 ± 49
(trans)[9R-MP]Z	nd	3 ± 3	47 ± 47	nd
[9R-MP]DHZ	1,010 ± 605	32 ± 16	373 ± 189	676 ± 236
[9R-MP]iP	163 ± 163	nd	189 ± 145	86 ± 86
(trans)(OG)Z	nd	15	nd	nd
(OG)DHZ	nd	nd	nd	nd
(cis)(OG)[9R]Z	144	123	107	nd
(trans)(OG)[9R]Z	nd	8	nd	nd
(OG)[9R]DHZ	nd	nd	nd	nd
Total	2,093 ± 919	935 ± 867	1,545 ± 568	1,662 ± 698
% cis-CK	40.1	85.3	58.6	48.9

[a] nd, Not detectable.

fruits, but their concentrations were variable during this 10-d period and generally were lower than the other groups of CK.

Accumulation of CK in the Tissues of Developing Fruits after Podset

As the fruits grew they accumulated CK up to 46 DPA, but the total present declined markedly after 46 DPA and by physiological maturity at 77 DPA had fallen to a level less than 10% of the peak accumulation (Fig. 2A). The sharp increase in total CK between 40 and 46 DPA was due to accumulation in the seed coat and, especially, in the liquid endosperm. Although a significant level of CK was recovered from the developing embryo at 46 DPA, its content declined from this time onward as the cotyledons began to expand and fill the endosperm space. The very large decline in CK in fruits between 46 and 61 DPA was almost entirely accounted for by the disappearance of the endosperm. Although the liquid endosperm accounted for only a very small proportion of the mass of the developing fruit, it contained close to 80% of the total CK present at 40 DPA (Fig. 2B). The pod wall accumulated as much as one-third of the total CK early in development, but this declined throughout growth and at the period of maximum CK accumulation it was less than 5% of the total.

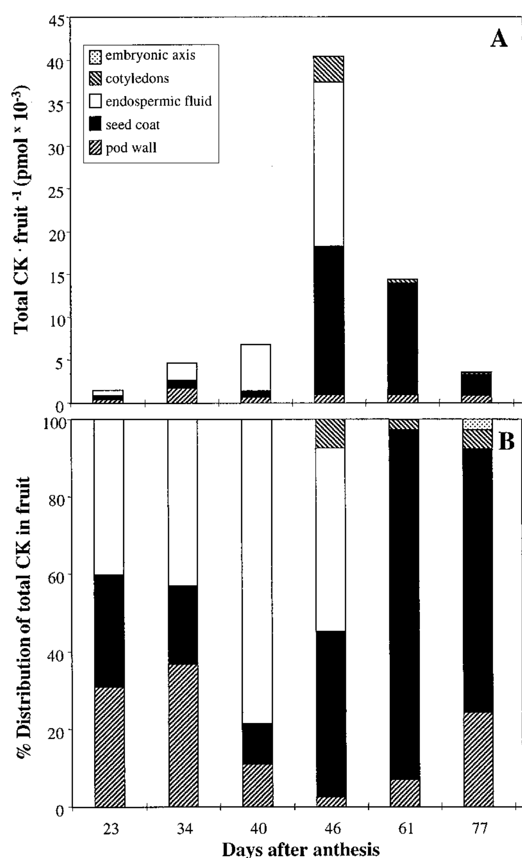

Figure 2. Total CK (A) and their percentage distribution (B) in component tissues of developing fruits at floret positions 1 to 3 (see Fig. 3) on the mainstem raceme of white lupine at intervals after the establishment of podset and up to physiological maturity at 77 DPA. Total CK were the sum of each form shown in Table II expressed on a per fruit basis.

At different times during fruit development 18 different forms of CK were detected in the component tissues (Table II). In general the pod wall showed a spectrum of CK that differed from the composition in seed tissues. Even early in development, when the content of CK was highest, there was relatively little of the major forms found in the seeds—dihydrozeatin (DHZ), trans-[9R]Z, and DHZ riboside ([9R]DHZ)—present in the pod, and the cis-isomers (average, 39%), nucleotides, and OG accounted for the majority throughout (Table II). Initially the cis-isomers also accounted for a significant proportion of CK in the seed coat, but this declined and became negligible as the fruits approached maturity. The major CKs in seed coats were trans-isomers, DHZ, and [9R]DHZ. By far the highest concentrations of CK throughout development were found in the endosperm, reaching values above 0.6 μmol g^{-1} fresh weight with negligible levels of the cis-isomers at each sampling time during the life of the endosperm (Table II). Although the very high concentrations recorded for endosperm occurred in part as a result of the diminishing volume at 40 DPA, and more obviously at 46 DPA, this compartment nevertheless continued to accumulate CK (Fig. 2A). Compared to the endosperm, the concentrations in the growing embryo were quite low (Table II) and, although initially the proportion as cis-isomers was similar to that in the endosperm, the embryonic axis and to a lesser extent the cotyledons had accumulated significant concentrations of cis-forms by physiological maturity.

Xylem and Phloem CK

Although both the levels and spectrum of CK recovered from xylem and phloem exudates were variable, there were fewer forms present in the transport fluids (Table III) than in the extracts of seed tissues (Table II). However, over the 77-d period of sampling the average CK contents of xylem and phloem were similar (55 $\pm$ 15 pmol mL^{-1} in xylem and 45 $\pm$ 7 pmol mL^{-1} in phloem; Table III). Up to 10 DPA both xylem and phloem collected from the raceme at the base of the inflorescence contained principally cis-[9R-MP]Z with the consequence that cis-isomers accounted for almost all of the CK present in these channels at a time when pods were setting. However, from 10 DPA onwards the proportion as cis-isomers declined to negligible levels in both fluids. Later during fruit development, DHZ nucleotide ([9R-MP]DHZ), [9R]DHZ, and trans-[9R]Z predominated in xylem, and [9R-MP]DHZ and [9R]isopentenyladenosine ([9R]iP) predominated in phloem. Smaller

Table II. *CK composition of the component tissues of developing fruits of white lupine at intervals after the establishment of podset and up to physiological maturity at 77 daa*

No endogenous (cis)Z or (cis)(OG)Z was observed in any of the tissues or sampling times, although there were good recoveries of corresponding ^{2}H-internal standards.

CK	Pod Wall DPA						Seed Coat DPA						Endospermic Fluid DPA				Cotyledons DPA			Embryonic Axis DPA	
	23	34	40	46	61	77	23	34	40	46	61	77	23	34	40	46	46	61	77	61	77
	pmol cytokinin g^{-1} fresh wt																				
(trans)Z	97	8	2	8	1	nd[a]	28	50	22	248	134	4	1,558	1,208	1,583	23,692	972	14	1	nd	nd
DHZ	85	nd	nd	5	nd	1	590	191	193	166	168	86	1,060	1,076	1,397	6,669	718	nd	nd	nd	nd
(cis)[9R]Z	173	132	75	49	2	51	300	216	87	351	18	1	189	171	572	14,364	443	2	15	5	507
(trans)[9R]Z	33	14	15	79	6	15	431	558	204	4,737	2,010	20	7,323	10,910	24,597	239,414	9,054	30	4	9	56
[9R]DHZ	16	7	11	29	11	31	585	934	585	3,932	3,674	1,029	5,004	6,604	16,824	109,083	4,686	119	8	37	34
iP	nd	nd	nd	nd	nd	nd	nd	nd	nd	15	nd	14	nd	nd	nd	nd	nd	3	nd	nd	nd
[9R]iP	44	nd	nd	nd	2	3	120	nd	nd	356	82	1	nd	nd	nd	29,508	nd	1	nd	nd	nd
(cis)[9R-MP]Z	18	7	31	10	20	3	27	24	11	16	27	1	860	379	102	598	119	2	11	nd	95
(trans)[9R-MP]Z	55	171	22	4	11	2	48	59	12	428	158	10	1,591	492	149	4,731	966	11	5	nd	54
[9R-MP]DHZ	46	80	14	8	84	6	81	64	55	377	46	307	576	135	151	2,873	645	85	35	nd	109
[9R-MP]iP	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	32	nd	nd	nd	nd	28	nd	nd	nd	nd	33
(trans)(OG)Z	nd	nd	nd	nd	nd	5	50	56	25	181	63	12	nd	678	1,196	15,729	nd	nd	nd	142	nd
(OG)DHZ	nd	11	4	7	2	10	285	218	106	24	59	15	110	nd	139	948	nd	3	1	118	26
(cis)(OG)[9R]Z	112	95	62	16	2	nd	236	150	72	274	nd	2	175	276	468	5,835	167	nd	nd	85	nd
(trans)(OG)[9R]Z	1	4	nd	2	4	4	122	117	108	2,642	121	36	3,849	4,566	4,746	167,522	nd	nd	nd	nd	nd
(OG)[9R]DHZ	8	2	2	61	7	49	218	nd	nd	nd	771	nd	nd	nd	nd	nd	nd	43	19	nd	4
Total	689	531	238	276	151	178	3,121	2,638	1,479	13,748	7,363	1,539	22,295	26,496	51,924	620,994	17,770	313	99	396	918
% cis-CK	43.9	44.0	71.0	27.0	16.0	30.1	18.1	14.8	11.5	4.7	0.6	0.3	5.5	3.1	2.2	3.3	4.1	1.2	27.0	22.8	65.5

[a] nd, Not detectable.

amounts of the side chain glucosides were present in the latter half of development, especially in phloem.

DISCUSSION

Identification and Quantification of CK in Developing Lupine Seeds

Earlier studies have analyzed the CK complement of developing seeds of white lupine (Davey and van Staden, 1979), *Lupinus angustifolius* (Zhang and Letham, 1990), and *Lupinus luteus* (Summons et al., 1981). Although no one has documented the presence of cis-forms, they indicate similar composition and concentrations of some of the other forms of CK to those described here for white lupine. Zhang and Letham (1990) used radio-immunoassay to measure CK once the liquid endosperm had disappeared, and their results are comparable to the data shown here for seeds at physiological maturity (77 DPA; Table II). Two groups of CK forms, the ribosides and nucleotides, were predominant, with CK nucleotides as the major form in the embryo and [9R]DHZ the highest in the seed coat. It is interesting that Zhang and Letham (1990) questioned the "exceptionally high" concentrations in seeds from which the endosperm had largely disappeared in the study of CK in white lupine by Davey and van Staden (1979). However, the very sharp transient increase both in concentration and accumulation (Table II; Fig. 2) corroborates the bioassay data of Davey and van Staden (1979) since we found CK levels were greatest once the endosperm had begun to decrease in volume (from 46–61 DPA). Zhang and Letham (1990) studied more mature seed and apparently overlooked this transient accumulation.

Summons et al. (1981) provide the only other study of CK from developing lupine seeds that used GC-MS. In immature *L. luteus* seeds, they detected a similar profile to the endospermic fluid or seed coats at 40 to 46 DPA of the present study but did not report the presence of cis-forms. The ribosides and a CK-OG were predominant and, although the concentrations were relatively high, they were still 100 times lower than those described here for white lupine. Again the levels of CK in more mature *L. luteus* seeds were very low and similar to those of white lupine seeds at 77 DPA (Table II).

A recent study of CK accumulation in developing seeds of another temperate grain legume, chickpea (*Cicer arietinum*), highlighted the prominence of cis-isomers both during early podset and later during grain filling (Emery et al., 1998a). As was the case in white lupine (Table II), very high transient concentrations of CK were recorded for the endosperm followed by a sharp decline as the cotyledons expanded and filled to physiological maturity. In chickpea, cis-[9R-MP]Z was the single most abundant CK and, during the early phase of seed development when cells in the embryo were dividing, accounted for

Table III. *CK composition of transport fluids collected from white lupine*

Xylem exudate was collected as bleeding sap from the root system after detaching the shoot. Phloem was collected from incisions in the vasculature of the raceme (0–10 DPA) or from the vasculature at the fruit tip (23–77 DPA). Although good recoveries of ^{2}H-internal standards were observed after purification, no endogenous (cis)Z, [9R]iP, (trans)[9R-MP]Z, [9R-MP]iP, (cis)(OG)Z, (trans)(OG)Z, or (OG)DHZ was detected at any of the growth stages. Likewise, no endogenous (cis)Z, (trans)Z, DHZ, (trans)[9R-MP]Z, [9R-MP]iP, or (cis)(OG)Z was detected at any growth stage in the phloem.

CK	DPA									Mean
	0	4	8	10	23	34	40	46	77	
	pmol cytokinin mL^{-1} sap									
Xylem										
(trans)Z	nd[a]	nd	nd	nd	7.7	nd	nd	3.3	nd	1.2 ± 0.9
DHZ	nd	nd	nd	nd	1.1	nd	nd	nd	nd	0.1 ± 0.1
(cis)[9R]Z	1.4	2.8	2.3	4.3	1.3	0.4	nd	1.9	nd	1.6 ± 0.5
(trans)[9R]Z	0.3	0.3	nd	nd	nd	nd	nd	15.2	13.0	3.2 ± 2.2
[9R]DHZ	1.1	0.8	0.3	8.8	0.8	nd	17.9	18.7	18.7	7.5 ± 3.1
iP	nd	nd	nd	nd	44.6	nd	nd	nd	nd	5.0 ± 5.3
(cis)[9R-MP]Z	21.4	25.9	nd	119.6	nd	nd	nd	2.2	nd	18.8 ± 13.8
[9R-MP]DHZ	nd	nd	nd	nd	nd	nd	40.4	25.5	56.0	13.6 ± 7.7
(cis)(OG)[9R]Z	na[b]	na	na	na	16.3	nd	nd	nd	1.3	3.5 ± 3.2
(trans)(OG)[9R]Z	na	na	na	na	6.5	nd	nd	5.8	nd	2.4 ± 1.5
(OG)[9R]DHZ	na	na	na	na	3.3	3.0	nd	nd	0.9	1.4 ± 0.7
Total	24.3	29.8	2.6	132.7	81.5	3.4	58.3	72.5	90.0	55.0 ± 15.4
% cis-CK	94.2	96.2	88.9	93.4	21.5	11.6	0.0	5.6	1.5	43.4 ± 16.0
Phloem										
(cis)[9R]Z	4.0	4.0	2.8	1.1	1.3	nd	nd	0.6	1.9	1.7 ± 0.6
(trans)[9R]Z	2.8	nd	nd	nd	nd	nd	nd	1.0	0.6	0.5 ± 0.3
[9R]DHZ	3.1	0.6	5.9	0.8	4.5	2.3	nd	1.5	4.9	2.6 ± 0.7
iP	7.9	nd	3.9	nd	nd	nd	nd	nd	nd	1.3 ± 1.0
[9R]iP	nd	nd	2.7	nd	nd	nd	20.1	12.3	5.9	4.5 ± 2.5
(cis)[9R-MP]Z	24.6	23.8	67.3	29.7	nd	nd	nd	8.9	nd	17.2 ± 7.9
[9R-MP]DHZ	nd	nd	nd	nd	nd	55.2	16.1	10.6	nd	9.1 ± 6.5
(trans)(OG)Z	na	na	na	na	nd	nd	nd	nd	32.0	6.4 ± 6.4
(OG)DHZ	na	na	na	na	nd	nd	nd	nd	5.1	1.0 ± 1.0
(cis)(OG)[9R]Z	na	na	na	na	9.2	nd	nd	nd	nd	1.8 ± 1.8
(trans)(OG)[9R]Z	na	na	na	na	nd	nd	nd	1.8	2.2	0.8 ± 0.5
(OG)[9R]DHZ	na	na	na	na	1.9	2.5	3.8	3.0	8.5	4.0 ± 1.2
Total	42.5	28.4	82.7	31.7	16.8	60.0	40.0	39.7	61.0	44.8 ± 7.1
% cis-CK	67.4	98.0	84.8	97.3	62.4	0.0	0.0	23.8	3.2	46.4 ± 14.8

[a] nd, Not detected. [b] na, Not analyzed.

around 70% of total CK. This was also the case in xylem sap of chickpea (81% CK as cis-[9R-MP]Z) collected from the base of the stem in the same way as from lupine. The study with chickpea indicated considerable variability in both level and spectrum of CK forms in seed tissues at different stages of development, and the present data from white lupine are similar in this respect. The peak concentration of CK in the endosperm at 46 DPA was more than 6,000 times the level in cotyledons at physiological maturity (Table II). Although the proportion of CK as cis-isomers in seed tissues of lupine was much lower than that found in chickpea seeds, cis-forms of the free-bases, ribosides, nucleotides, and side chain glucosides were routinely detected. Other lupine species (*L. angustifolius* and *Lupinus mutabilis*) also show this range of cis-forms (R.J.N. Emery, Q. Ma, and C.A. Atkins, unpublished data). Thus, questions raised about the source of cis-CK and their bioactivity in chickpea (Emery et al., 1998a) apply equally for lupines, especially during the initial stages of podset and embryogenesis.

van Staden et al. (1982) suggested that the high concentrations of CK in endosperm of lupine augment seed growth by promoting cell division in the embryo. In general CK accumulation in seeds of both cereals and legumes has been associated with embryogenesis and the early period of seed filling (Rock and Quatrano, 1995). Although this may be true, the highest accumulation in white lupine actually occurred after maximum endosperm volume was reached and the embryo had begun to expand. Thus the transient high concentrations coincided with the onset of seed filling, and it is conceivable that CK fulfilled a role in enhancing sink strength in some way other than promoting cell division in the embryo. An alternative is

that of Clifford et al. (1986), who demonstrated that benzylaminopurine (BAP) applied directly to excised seed coats of developing *Phaseolus vulgaris* seeds immediately stimulated unloading of ^{14}C-labeled assimilate. However, more recent molecular evidence indicates that CK might exert its effect on maintaining cell division within the developing embryo of fava bean through a sugar signaling mechanism (Herbers and Sonnewald, 1998). CK-enhanced coordinate expression of invertase and a hexose transporter in Arabidopsis (Truernit et al., 1996) and *Chenopodium rubrum* (Ehness and Roitsch, 1997) support this idea.

Changes in CK Content in Relation to Podset

Abortion and abscission of a large proportion of flowers following their fertilization is a common feature of reproductive development among legumes. A wide range of environmental factors can influence the extent of "flower shedding," and as a consequence the yield potential of many pulses may not be realized. Because of its agronomic importance, the subject has received considerable attention. Theories as to the cause include hypotheses based on competition for nutrients (Pate and Farrington, 1981; Brun and Betts, 1984) and those involving hormonal influences that promote the set of pods at some floret positions or inhibit podset at other floret positions (van Steveninck, 1959). Although roles for CK, gibberellin, indoleacetic acid, and abscisic acid have been suggested in early embryogenesis (for review, see Rock and Quatrano, 1995), a more specific function for any of these in regulating podset has not been clearly defined. Exogenous application of CK a few days before or even at anthesis, particularly to sepals enclosing the ovary of *L. angustifolius* flowers that are destined to abort, reverses their fate and they set and develop pods instead (Atkins and Pigeaire, 1993). BAP was used routinely, but [9R]Z and [9R]DHZ were equally effective. Although a response to exogenous application of CK does not necessarily indicate a function for endogenous CK in determining podset, applying BAP after anthesis was less effective and had no effect on podset once the flowers had begun to senesce (about 5 DPA; Dracup and Kirby, 1996). One obvious change in the CK composition between anthesis and 4 DPA was the significant transient increase in the proportion of cis-forms in ovaries in the two zones where flowers were destined to abort (Table I), whereas in those destined to set pods the proportion as cis-forms did not change. If it can be assumed that the "active" forms in lupine ovary tissue are among the free bases and ribosides, then the ratio of cis-forms:trans-forms in this group changes progressively from more than 17:1 at anthesis to less than 1:1 by 10 DPA at positions 1 to 3, whereas at positions 4 to 6 there is a progressive decline from 16:1 to 3:1, and at positions 7 to 10 the ratio remains high throughout the 10-d period ranging from 7:1 to more than 19:1 (derived from Table I).

Dybing et al. (1986) concluded that in soybean, ovary abortion is initiated by events that slow growth at or within 1 DPA. Thus changes in abundance of cis- and trans-isomers seen here for white lupine could be a consequence of earlier events rather than a cause of flower abortion. Nevertheless, cis-isomers may well act as competitive inhibitors of processes that involve the binding of or reaction with trans-isomers (Kuraishi et al., 1991), and in this way they may exert a negative effect on embryogenesis. Bassil et al. (1993) partially purified a Z cis-trans isomerase from the endosperm of developing *P. vulgaris* seeds, and it is tempting to speculate that regulation of the expression or activity of an equivalent enzyme in lupine might be one of those "early events."

The fact that ovaries destined to abort and abscise at the highest floret positions (7–10) had essentially ceased growth by 4 DPA and did not accumulate CK is consistent with reduced translocation of assimilates and possibly also of CK. Both in *L. angustifolius* (Pate and Farrington, 1981) and soybean (Brun and Betts, 1984) the ability of flowers to attract ^{14}C-labeled assimilates declines to almost zero at anthesis. Prior to anthesis unopened flower buds have demonstrable sink strength, and in those destined to set pods this ability is regained at about 4 DPA. However, in ovaries destined to abort, the ability to attract assimilates does not reappear after anthesis. Pate and Farrington (1981) showed that phloem exudation from pedicels of flowers destined to set pods continued after anthesis; whereas in those destined to abort, exudation ceased, and they speculated that phloem translocation was compromised. At anthesis vascular tissue at the site of the abscission zone in the pedicel is not well developed and does not further develop after anthesis in flowers that will abort (Clements, 1996). On the other hand, in flowers that set pods or to which CKs have been applied earlier (Atkins and Pigeaire, 1993), there is rapid vascular development. The events that lead to abscission of aborted ovaries in lupines are also initiated soon after anthesis, and the differentiation of an abscission zone in the pedicel is closely associated with a loss of translocation activity (Clements and Atkins, 1999).

Transport Fluids and the Translocation of CK

The significance of long-distance transport of CK in plant development is far from clear (for review, see Letham, 1994). Even the forms of CK that are mobile in xylem and phloem and their origin(s) are uncertain. The information on composition is fragmentary (Hoad, 1995) at best, particularly for phloem, being restricted to single stages of growth and in most cases lacking the rigor of unambiguous chemical identification of the forms. Lupines are unique among grain legumes in providing exudates from both xylem and

phloem. It is not surprising, therefore, that a number of studies have analyzed the CK contents of these transport fluids (Davey and van Staden, 1978; Summons et al., 1981; Jameson et al., 1987; Taylor et al., 1990). However, each of these analyses suffers from the shortcomings cited above. Taylor et al. (1990) collected both xylem and phloem of white lupine throughout plant development and during the growth of fruits but used bioassays to detect "CK-like substances." Although the identification of CK species was ambiguous, there was significant variation in concentration in both translocation channels, especially in xylem. One of only two detailed analyses of phloem composition using GC-MS was carried out by Taylor et al. (1990), but unfortunately it combined xylem and phloem sap samples for a single time (approximately 60 DPA) and only identified [9R]Z and DHZ as constituents. A second study of phloem managed to obtain GC-MS full scans from castor bean but analyzed only two CK from a single, undefined sampling time (Kamboj et al., 1998). Although they are restricted to a single site on the plant and to a 77-d period of reproductive development, the data presented in this study of white lupine represent the most detailed documentation of CK in transport fluids, and especially in phloem exudates, for any species.

It is clear that the spectrum of CK forms in translocation channels, particularly in phloem, is far from constant (Table III), highlighting the danger in reaching conclusions about the mobility of growth regulators based on exudate samples collected at just one stage of growth. Although some of the variation could reflect differences in the dynamics of water movement or the nitrogen status of the plant (Beck and Wagner 1994; Sakakibara et al., 1998) at the sampling times chosen, they could also reflect developmentally conditioned changes in CK synthesis and translocation. The concept that CK constitute an important root-shoot signal derives in part from the many analyses of xylem that have shown environmentally induced changes in CK level or changes that are correlated with developmental events in the shoot (Letham, 1994). Recent evidence linking soil nitrogen status and supply to CK levels and transcription of a CK-inducible response regulator (ZmCip1; Sakakibara et al., 1998) supports this concept. The forms of CK that constitute this signal in planta have not been defined, but the wide variation in forms described in this study might reflect a number of "signals."

Beck and Wagner (1994) found eight different CK in xylem sap of the stinging nettle (*Urtica dioica*), but because six of these ([9R-MP]Z, DHZ, [9R]DHZ, [9R-MP]DHZ, [9R]iP, and iP nucleotide) were extraordinarily variable, they concluded that only Z riboside and Z were translocated forms. The others, which individually accounted for from 0% to 10% of the total CK, were regarded as "impurities" derived from damaged cells. In the present study the initial root bleeding xylem sap was discarded to minimize the possibility of contamination. On the other hand all the phloem that exuded was collected, and it is possible that some of the CK complement came from damaged cells or from the apoplast exposed to high sugar concentrations. There have been no detailed analyses of phloem exudate collected from lupine using aphid stylets, so a comparison with sap from a non-wound source at the same site is not possible. However, spontaneous phloem exudate collected from the proximal pedicel abscission zone face of *L. mutabilis* (Atkins, 1999) contained as little as 9 pmol CK mL^{-1} exclusively as cis-Z riboside, whereas exudate collected from incisions in the stems and supporting racemes contained mainly isopentenyl adenosine ([9R]iP) (54 pmol mL^{-1}).

In both xylem and phloem collected during the first 10 DPA the cis-forms, especially cis-[9R-MP]Z, were predominant, and the developing ovaries also showed a relatively high proportion of CK as cis-isomers. The increment in total CK over this 10-d period was quite small (around 30 pmol; Fig. 1B), and it is reasonable to conclude that much of the CK complement found in these organs was due to translocation. At this time both xylem and phloem would be expected to have contributed about equally to the nutrition of the ovaries (Pate et al., 1977). If this is so, then the tissue composition is consistent with activity of a nucleotidase or phosphatase to yield cis-[9R]Z, a reductase to yield [9R-MP]DHZ with loss of inorganic phosphate and Rib (through nucleosidase activity) to form [9R]DHZ and DHZ, respectively. As noted earlier, it is possible that trans-[9R-MP]Z and trans-[9R]Z could be generated from cis-[9R-MP]Z and cis-[9R]Z, respectively, by a cis-trans isomerase, although it has not been established that the enzyme can catalyze the isomerization of substrates other than Z (Mok and Martin, 1994).

After the pods had set at floret positions 1 to 3 (i.e. after 10 DPA), xylem contained mainly [9R-MP]DHZ, [9R]DHZ, and at late stages of development trans-[9R]Z (Table III). Jameson et al. (1987) also reported [9R]DHZ and [9R]Z as major constituents of root bleeding xylem sap of *L. angustifolius*, and Taylor et al. (1990) confirmed [9R]Z by GC-MS but in combined xylem/phloem samples. During this period the CK composition of fruit phloem was very variable, with [9R-MP]DHZ, [9R]iP, or a number of side chain glucosides predominating at different sampling times (Table III). As noted above, the reasons for such variation are not known; however, once the major sites for phloem loading of CK have been defined it may shed more light on interpreting changes in CK levels or composition in phloem. Labeling studies (Jameson et al., 1987; Zhang and Letham, 1990) in which high specific activity [^{3}H][9R]Z or [^{3}H][9R]DHZ was added to the transpiration stream of the main or lateral shoots of *L. angustifolius* indicated

significant radial movement and accumulation of label as [^{3}H]CK nucleotides in stems. This is perhaps not too surprising since ^{14}C-labeled basic amino acids (e.g. Arg) added to the transpiration stream of lupine are also largely abstracted in the stems and retained in the vasculature of petioles and leaflets (McNeil et al., 1979). Letham (1994) has speculated that stems function to temporarily store, modify, and contribute CK to phloem in the shoot, and Taylor et al. (1990) provided some evidence consistent with short-term xylem-phloem exchange of CK in upper stems of lupine. Summons et al. (1981) recovered [^{3}H]DHZ in fruit tip phloem of lupines following [^{3}H][9R]Z applied via root xylem, and both Davey and van Staden (1981) and Atkins and Pigeaire (1993) showed that a small proportion of labeled CK applied to leaves of lupine can be recovered in axillary branches and raceme (but not floral or fruit) tissues, presumably due to phloem translocation. However, phloem loading is highly solute specific, and it is likely that the forms of CK used in such studies might be critical to the extent of their loading. Overall the picture appears to be one of relatively low phloem mobility, and if indeed there is significant contamination of exudate as a consequence of wounding (Atkins, 1999), then in planta phloem may contribute relatively little CK to sink organs.

The CK Economy of Developing Lupine Fruits

Estimates for the translocation of CK in both xylem and phloem to developing fruits (Table IV) were made using the mean concentrations of total CK in these exudates (Table III) together with a previously published (Pate et al., 1977) quantitative water economy of the fruits. When compared to the increments in CK accumulation over six periods of development, it is clear that most of the CK recovered in fruits was not due to translocation. Even if the CK levels in phloem were elevated due to contamination, then phloem delivery would be overestimated. Similarly, the CK content in root bleeding exudate may not be the same as that in the transpiration stream at the top of the shoot serving the developing fruits. The concentration of solutes in exudate due to root pressure is usually much higher than that of tracheal sap collected by vacuum or pressure displacement from stems. A preliminary study with *L. angustifolius* showed that tracheal sap at the base of the stem contained about one-half the CK level of sap collected by root pressure and at the top of the stem was concentrated by about 2-fold compared to the base (R.J.N. Emery and C.A. Atkins, unpublished data). Progressive concentration of solutes in the transpiration stream by xylem-xylem exchange is a feature of the distribution of nitrogen in the white lupine shoot (Layzell et al., 1981), and this appears also to be the case for CK. Despite these reservations about the CK levels in both phloem and xylem, there would have to be a very large adjustment to have any impact on the CK budget in Table IV. It is thus very likely that there is extensive synthesis in the fruit tissues, possibly in the seed coat and endosperm. The large increments of CK in the lupine seed in the period 41 to 46 DPA were apparently almost entirely the consequence of synthesis in situ. Although it is difficult to envisage this being the result of external colonization by bacterial symbionts or of synthesis by symbiotic bacteria present within the seed tissues (Holland, 1997), such possibilities cannot be dismissed. This study did not attempt to control the presence of bacteria either within or on lupine fruit tissues, and at present their occurrence and ability to synthesize CK at these sites remains unknown.

The significant negative CK increment from 47 to 61 DPA (Table IV) reflects the loss of the endosperm and the fact that the extraordinarily large amount of CK that it contained simply disappeared from the fruit (Fig. 2A). From 62 to 77 DPA, the net loss of CK was from the seed coat (Fig. 2A). These substantial losses of CK either were due to degradation or were the result of translocation out of the fruit. In cowpea (*Vigna unguiculata* [L.] Walp.), the second half of fruit development is characterized by a diurnally reversing xylem flow from the fruits to the subtending peduncle and upper leaves (Pate et al., 1985), appar-

Table IV. *Estimates for the translocation of CK in both xylem and phloem to developing fruits of white lupine using the mean concentrations of total CK in these exudates together with a previously published (Pate et al., 1977) quantitative water economy of the fruits*

Comparisons are made to the increments in CK accumulation over six periods of development.

Item		DPA						Notes
		0 to 23	24 to 34	35 to 40	41 to 46	47 to 61	62 to 77	
1	Xylem water intake (mL)	2.5	6.2	3.3	3.3	6.7	3.7	Data calculated from Pate et al. (1977)
2	pmol CK ml xylem^{-1}	55.0	55.0	55.0	55.0	55.0	55.0	Mean value from Table III
3	Total pmol CK to fruit in xylem	137.0	339.9	178.8	178.8	366.3	204.1	Calculated as item 1 × item 2[a]
4	Phloem water intake (mL)	1.1	1.7	2.4	2.4	8.4	1.5	Data from Pate et al. (1977)
5	pmol CK mL phloem^{-1}	45.0	45.0	45.0	45.0	45.0	45.0	Mean value from Table III
6	Total pmol CK to fruit in phloem	50.9	76.1	109.8	109.4	378.5	67.1	Calculated as item 4 × item 5[a]
7	Total pmol CK from translocation	187.8	416.0	288.6	288.1	744.8	271.1	Item 3 + item 6
8	pmol Change in total CK in fruit	1,491.6	3,209.8	2,069.2	33,627.0	−26,003.5	−10,801.0	Data calculated from Table II
9	% CK in fruit from translocation	13	13	14	1	−3	−3	Item 7/item 8 × 100

[a] Assumes constant CK over the period and diurnally.

ently because the import of water in phloem exceeds transpiration (Peoples et al., 1985). Although reverse flow in xylem has not been demonstrated for white lupine, it could provide a route for CK to cycle back to the parent plant. In this way the seed would be not only a major site of CK synthesis but also a major source of these regulators for the plant as a whole, potentially exerting some regulatory "influence" on events elsewhere.

MATERIALS AND METHODS

Plant Material

White lupine (*Lupinus albus* L. cv Kiev mutant) plants were nodulated with *Bradyrhizobium* sp. strain WU 425 and were grown from May to December (the normal growing season for lupines in Western Australia) in sand culture with a one-fourth-strength Hoagland solution free of combined nitrogen in a greenhouse under natural light.

During the first 10 DPA, fertilized ovaries were collected from three zones on the mainstem inflorescence (Fig. 3). The raceme on lupine may develop as many as 40 flowers that are self-fertilized progressively from the base at intervals of 1 to 2 d (Dracup and Kirby, 1996). Flowers at the lowest floret positions (1–3) will set pods with close to 100% frequency (Pigeaire et al., 1992), whereas those at positions 4 to 6 will abort and abscise in 30% to 40% of cases. At positions 7 to 10 all flowers are destined to abort by about 10 DPA. Each of three replicate samples comprised 14 to 20 ovaries collected from five to seven plants.

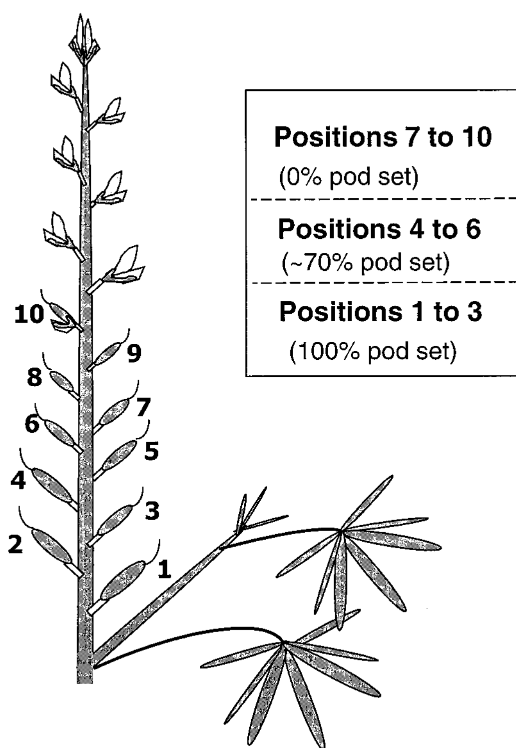

Figure 3. Mainstem flowering raceme of white lupine showing the numbering system of floret positions. Frequency of successful pod set is indicated for the three zones from which fertilized ovaries were collected during the first 10 DPA.

At sampling times from 23 to 77 DPA, fruits were collected exclusively from the main-stem raceme at floret positions 1 to 3 (Fig. 3). At 23 DPA collections comprised tissues from 27 seeds; at 34 DPA they comprised tissues from 15 seeds, and for the other sampling times they comprised tissues from six to 13 seeds. By 77 DPA the fruits had reached physiological maturity—seed filling was complete, the cotyledons had lost most of their chlorophyll, and the pod was beginning to desiccate (Pate et al., 1977; Dracup and Kirby, 1996). Tissues of fruits collected from 23 to 40 DPA were separated into pod wall, seed coat, and endospermic fluid. Endospermic fluid was extracted using a pipette with a fine 5-μL pipette tip inserted into a slit made in the seed coat (Atkins et al., 1975). At 46 DPA the embryo was dissected from the seed and extracted separately. After 46 DPA the endosperm had disappeared and embryos were sufficiently large to separate the cotyledons from the embryonic axis. Depending on the developmental stage, samples of endospermic fluid were from 75 to 270 mg, seed coat was from 0.75 to 3.0 g, embryo was 0.4 g, cotyledons were 2.8 to 4.3 g, and embryonic axis was from 140 to 250 mg fresh weight. All plant tissue samples were stored frozen at $-80°C$ before extraction.

Collection of Saps

Phloem sap was collected in sterile glass microcapillaries as it exuded from shallow incisions made in the raceme below floret positions 1 to 3 up to 10 DPA and in the tips of pods that were subsequently harvested for tissue analyses at 23 to 77 DPA. Following phloem collections xylem sap was collected by decapitating plants about 2 cm above where the stem exited the sand. The cut stump was rinsed with deionized water, and initial root pressure exudate was discarded. Silicone tubing was attached to the cut stumps, and exudates were collected over the following hour, snap frozen in liquid nitrogen, and stored. Collections were made from five to seven plants for xylem and from 15 to 20 plants for phloem, and they were pooled to obtain 0.5 to 1 mL of both types of sap for analysis.

Tissue Extraction

Extraction and purification followed protocols that have been proven not to degrade CK nucleotides or tRNA and that do not cause artifacts of CK isomerization (Emery et al., 1998a). Five nanograms (anthesis to 10 DPA) or 60 ng (23–77 DPA) each of [$^2H_6$]iP, [$^2H_6$][9R]iP, (trans)[$^2H_5$]-Z, [$^2H_3$]DHZ, (trans)[$^2H_5$][9R]Z, and [$^2H_3$][9R]DHZ and 10 ng (anthesis to 10 DPA) or 80 ng (23–77 DPA) each of [$^2H_6$]iP nucleotide, (trans)[$^2H_5$][9R-MP]Z, and [$^2H_3$][9R-MP]DHZ (Apex Organics, Devon, UK) were added as internal standards. No extraction step was necessary for samples of transport or endospermic fluids, which were directly freeze-dried and purified in the same manner as tissue extracts.

Purification and Assay of CK

The residue from the extraction was passed through a sterene divinylbenzene (500 mg) strong cation exchange solid phase extraction column (Alltech Associates, Baulkham Hills, NSW, Australia) to separate CK nucleotides from nucleoside and free-base CK. The fraction containing the latter groups was passed through a 300-mg C_{18}, solid-phase extraction cartridge (Alltech Associates) and further purified by HPLC on a C_{18}, Alphabond column (300-mm length, 3.9-mm i.d., and 10-μm particle size, Alltech Associates). CK eluted from a 40-min linear solvent program from 5% to 30% acetonitrile in water adjusted to pH 7 with triethylammonium bicarbonate. Collected fractions were freeze-dried, and the residues were transferred in methanol to tapered glass vials for derivatization.

CK nucleotides recovered in the acetic acid wash of the strong cation exchange solid phase extraction columns were converted to nucleosides by incubation with alkaline phosphatase (Type III, Sigma, St. Louis, MO; 3.4 units in 1 mL of 0.1 M ethanolamine-HCl, pH 10.4), and resultant CK nucleosides were purified as outlined above.

The CK were permethylated as described earlier (Emery et al., 1998b), and an aliquot in ethyl acetate was analyzed by GC-MS. The GC (5890, Hewlett-Packard, Palo Alto, CA) was fitted with a BP5 capillary column (25 m, 0.22-mm i.d., and 0.25-μm film; Scientific Glass Engineering, Ringwood, VIC, Australia) linked to a Hewlett-Packard 5970 series mass selective detector. Ions for selected ion monitoring identification and those used for calculating endogenous CK levels together with details of retention indices to differentiate between cis- and trans-isomers are detailed in Emery et al. (1998a). Where necessary, corrections were made for the contribution of ^{2}H ions to ^{1}H ions (and vice versa).

Calculation of CK Delivery Rates from Transport Fluids to Seeds

Estimates of water delivery in xylem and phloem were based on a previous study (Pate et al., 1977) of the carbon, nitrogen, and water intake budget of developing fruits/seeds of white lupine. Concentrations of CK measured in xylem and phloem were multiplied by those estimates of water delivery to arrive at a CK delivery rate to the seeds. This was compared to the total CK that accumulated in the pod and seed tissues to determine the proportion of CK that might have been synthesized outside the fruit and delivered in translocation channels.

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