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ORIGINAL PAPER

Comprehensive hormone profiling of the developing seeds of four grain legumes

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Abstract
Key message **Developmental context and species-specific hormone requirements are of key importance in the advancement of in vitro protocols and manipulation of seed development.**
Abstract Improvement of in vitro tissue and cell culture protocols in grain legumes such as embryo rescue, interspecific hybridization, and androgenesis requires an understanding of the types, activity, and balance of hormones within developing seeds. Towards this goal, the concentration of auxin, cytokinin, gibberellin, and abscisic acid (ABA) and their precursors and derivatives were measured in the developing seeds of field pea (*Pisum sativum* L.), chickpea (*Cicer arietinum* L.), lentil (*Lens culinaris* Medik.), and faba bean (*Vicia faba* L.) from 4 days after anthesis until 8 days after reaching maximum fresh weight. The importance of developmental context (developmental time and space) is demonstrated in both the differences and similarities between species for hormone profiles, especially with regard to cytokinin and ABA biosynthesis during the embryo formation. Auxin and its conjugates are significant during the pattern formation stage of all legumes; however, IAA-Asparagine appears important in the *Vicieae* species and its concentrations are greater than IAA from the globular stage of embryo development on in multi-seed fruits. Finally, the significance of non-polar gibberellins during lentil seed development is highlighted.

Keywords Abscisic acid · Auxin · Chickpea · Cytokinin · Seed development · Faba bean · Field pea · Gibberellin · Lentil

Introduction

Seed growth involves the coordinated development of the diploid maternal ovule, triploid endosperm, and diploid embryo. In angiosperms, this process consists of four main phases: pattern formation, maturation, desiccation, and germination. Pattern formation involves the development of the embryo proper as well as endosperm and seed coat enlargement, and these processes require peaks in cytokinin and auxin. During maturation, the endosperm is absorbed by the embryo and seed reserves of carbohydrates, oils, and proteins are deposited. Changing ratios of abscisic acid (ABA) and gibberellin are linked to this process. During desiccation, the seed prepares for dormancy as it continues dehydration, finishes depositing reserves, and increases the concentration of ABA. The final stage of a seed's life is germination, when imbibition occurs, gibberellin concentrations increase, reserves mobilize, and vegetative growth resumes.

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Genomic and mutant studies have provided insight into the potential functions of auxin, cytokinin, ABA, and gibberellin during seed development. Cytokinin is linked to increased cell division, which is an active part of the pattern development phase. The most biologically active cytokinins are *trans*-zeatin (tZ), dihydro-zeatin (dhZ), and isopentenyladenine (2iP), whereas *cis*-zeatin (cZ) is considered to have weak or no activity (Bajguz and Piotrowska 2009; Sakakibara 2006). Nonetheless, the *cis*-isomers are reportedly the major forms of cytokinin in chickpea and pea seeds (Emery et al. 1998; Quesnelle and Emery 2007) and their lack of activity in standard bioassays may be due to customization of those assays for *trans*-isomer detection (Quesnelle and Emery 2007). Cytokinin form and activity are dependent on plant species, tissue, and developmental stage (Kieber and Schaller 2010). Cytokinins promote seed growth in legumes by enhancing sink strength either through promotion of cell division and metabolism of imported sucrose or increased phloem unloading in the seed coat (Emery et al. 1998, 2000; Quesnelle and Emery 2007). Although cytokinin is transported into the seeds of lupine, most cytokinin is synthesized in situ, probably in the endosperm (Emery et al. 2000; Miyawaki et al. 2004; Quesnelle and Emery 2007). In legumes, cytokinin concentrations are highest in the early endospermic fluid stage and fall when cotyledons expand (Emery et al. 1998; Toker et al. 2006).

Auxin is a major hormone during pattern formation that affects not only the embryo but also the size of the endosperm (Van Daele et al. 2010). Auxin gradients form the embryonic apical-basal axis, the shoot and root meristems, and the cotyledon organs (Braybrook and Harada 2008; Jenik and Barton 2005). In addition to the biologically active free form indole-3-acetic acid (IAA), auxin can be conjugated to sugars, amino acids, or peptides (Ljung et al. 2002). IAA conjugates are generally considered a reservoir for the maintenance of IAA homeostasis (IAA-leucine, IAA-glycine) or inactive (IAA-aspartic acid, IAA-glutamic acid) metabolites (Ljung et al. 2002; Teale et al. 2006). However, recent research indicates that the irreversible conjugation of IAA to asparagine (Asp) and glutamine (Glu) is not simply a means of inactivating IAA but that these conjugates have a separate signaling function (Rosquete et al. 2012). A separate signaling function for IAA-Asp is indicated by: (1) the production of IAA-Asp which appears to be an ancient pathway (Ludwig-Muller 2011); (2) the presence of enzymes in immature pea seeds to specifically make IAA-Asp (Ostrowski and Jakubowska 2011); and (3) the suggested independent activity of IAA-Asp (Ljung et al. 2002; Oetiker and Aeschbacher 1997; Pinto et al. 2011; Tam et al. 2000). The chlorinated form, 4-chloroindole-3-acetic acid (4-Cl-IAA), is ten times more active within *Fabaceae* species, which include field pea, lentil, and faba bean (Engvild et al. 1981; Park et al. 2010; Reinecke 1999; Simon and Petrasek 2011). 4-Cl-IAA is formed in the seed and transported to the pericarp, where it is involved in gibberellin biosynthesis and pericarp development (Ozga et al. 2009; Park et al. 2010), and is considered a transportable seed factor involved in coordinating early pea seed and pericarp growth (Reinecke 1999).

High concentrations of auxin trigger the production of bioactive gibberellin (GA_1, GA_3, GA_4, and GA_7) (Dorcey et al. 2009). In pea, gibberellin is important for the regulation of early seed development and cell expansion (Nadeau et al. 2011; Singh et al. 2010; Swain and Singh 2005; Weber et al. 2005), proper fruit set and development (Alabadi et al. 2009), and germination and seedling growth. Nadeau et al. (2011) found that bioactive gibberellin is involved in determining the rate of seed coat growth and sink strength during early seed development. GA_{53}, GA_{44}, GA_{19}, and GA_{20} are considered precursors of GA_1, whereas GA_{29} and GA_8 are the derivatives of this pathway; GA_{24} and GA_9 are the precursors of the GA_4 pathway and GA_{34} is the derivative (Yamaguchi 2008).

The ratio of ABA to gibberellin concentration is linked to embryo development and seed fill rate during the maturation phase (Alabadi et al. 2009; Liu et al. 2010; Weber et al. 2005), and ABA is necessary to proceed through seed maturation to desiccation tolerance and dormancy (Weber et al. 2005). ABA and gibberellin are initially from maternal sources (Liu et al. 2010; Nambara and Marion-Poll 2003) although there is flexibility in the pathway (Kanno et al. 2010). ABA is considered the bioactive form; however, 7′, 8′, and 9′-hydroxy ABA show hormonal activity in *Brassica napus* embryos (Jadhav et al. 2008). It can be inactivated by phytoisomerization (t-ABA), and major precursors (ABA-glucose ester) and derivatives [7′-hydroxy ABA, phaseic acid (PA), neo-PA and dihydrophaseic acid (DPA)] are discussed elsewhere (Hampson et al. 1992; Zaharia et al. 2005; Zeevaart 2003).

Although specific hormones throughout embryo development or hormone profiles at specific embryo developmental stages have been examined in some grain legumes (Emery et al. 1998, 2000; Toker et al. 2005, 2006; Weber et al. 2005), a comprehensive profiling of auxin, gibberellin, cytokinin, ABA, and their precursors and derivatives has not been conducted throughout the pattern formation and early maturation stages of legume embryos. Notably, recent advances in our ability to measure minute amounts of hormones and their metabolites have occurred; for example, liquid chromatography-electrospray ionization-tandem mass spectrometry (LC–ESI–MS/MS) has been applied to comprehensive hormone analysis (Kanno et al. 2010; Lulsdorf et al. 2013) and is suitable for the simultaneous quantification of several hormones. Precursors are important indications of the activity of the hormone

pathway not represented by the total amount of bioactive hormone concentration. Although understanding of the molecular mechanisms controlling seed development has vastly improved over the last few years (Ljung et al. 2002; Rosquete et al. 2012; Weber et al. 2005), the links between these mechanisms and hormonal control of the developmental pathway are not well understood.

The objective of this study was to compare the concentration of auxin, gibberellin, cytokinin, and ABA and their precursors and derivatives in the developing seeds of four grain legumes—chickpea (*Cicer arietinum* L.), lentil (*Lens culinaris* Medik.), pea (*Pisum sativum* L.), and faba bean (*Vicia faba* L.). These legumes are recalcitrant in tissue culture (Croser et al. 2006). An understanding of hormonal balance, as well as the activity of these hormonal pathways, within grain legumes is important for understanding all aspects of legume seed development and has direct applications to in vitro tissue and cell culture, specifically embryo rescue and interspecific hybridization.

Materials and methods

Plant growth conditions

Chickpea (CDC Xena), field pea (CDC April), and faba bean (Breeding line 219-18) (Table 1) were grown in University of Saskatchewan greenhouses located in Saskatoon, Canada (52°8′N/106°40′W). Natural light was supplemented with high pressure sodium lamps with an 18 h light/6 h dark photoperiod and growth temperatures of 22 ± 1 °C light/20 ± 1 °C dark. Chickpea and field pea were grown in Sunshine® Mix No. 4 and faba bean in Sunshine® Mix No. 3 (Sun Grow Horticulture, Vancouver, British Columbia, Canada), each at a planting density of five plants/11 L pot. Lentil (CDC Maxim) was grown in the University of Saskatchewan polyhouse and field in Sutherland series clay. The lentil flowers were tagged and seeds collected in July–August of 2012 during which the average temperature was (24 ± 2 °C day/12 ± 2 °C night) and the total precipitation was 251 mm (Environment Canada 2013).

Sample collection

Flowers of all species were tagged on the day of anthesis [0 days after anthesis (0 DAA)] when flowers were still closed and pollination imminent. Samples of developing pods were collected 4 days later and every 4 days thereafter until physiological maturity. Seed size and embryo development depended on the species and cultivar (Table 2). Staging of embryo development was done using a stereo microscope. Since the seeds from 4 to 8 DAA were too small for dissection of specific organs (e.g., embryo or endospermic fluid), hormone quantification was done on entire seeds for all growth stages. Seeds were dissected out using a stereo microscope and approximately 500 mg of fresh weight was collected for each sample. All material was kept on ice during dissection and then freeze-dried.

Immature seeds were collected from 5 to 10 pods/plant of 10 to 50 plants grown at the same time in a completely randomized design. Three sub-samples of this seed collection were used for hormone analysis. Statistical analysis was done using SAS 9.2. Least significant differences (LSD) at $\alpha = 0.05$ or mean ± standard error (SE) from three replicates are reported in nmol g^{-1} dry weight (DW).

Hormone analysis

Quantification of ABA, cytokinin, auxin, and gibberellin was conducted at the National Research Council of Canada-Saskatoon by ultra-performance liquid chromatography-electrospray ionization/multi-stage mass spectrometry (LC–ESI–MS/MS, http://www.nrc-cnrc.gc.ca/eng/solutions/advisory/plant_hormone.html) using deuterium-labeled internal standards. The procedure for the hormone profiling analysis is described in Lulsdorf et al. (2013) using an existing method (Chiwocha et al. 2003, 2005), with modifications to the accommodate analysis of 4-Cl-IAA (purchased from Ark Pharm, Inc. USA). In the absence of an appropriately labeled internal standard, 4-Cl-IAA was quantified using a calibration curve built on its response against d_3-IAA-Leu. Experiments were replicated three times and hormone content was calculated based on dry weight.

Table 1 Grain legume seed characteristics

	Seed weight (g/100 seeds)	Percentage of protein:oil:carbohydrate	No. of seeds per pod	Seed coat color	No. pods per peduncle
Chickpea (CDC Xena)	~45	~21:5:40–60	1–2	Kabuli—yellow cotyledons; clear seed coat	2
Lentil (CDC Maxim)	4	20:1:65	1–2	Reddish brown	2
Field pea (CDC April)	14	25:1:52	3–7	Brown–green	1–2
Faba bean (Breeding line 219-18)	35–50	23:1:56	3–7	Light brown/yellow	1

Table 2 Embryo developmental stage of chickpea (CDC Xena), lentil (CDC Maxim), field pea (CDC April), and faba bean (Breeding line 219-18) at 4-16 DAA

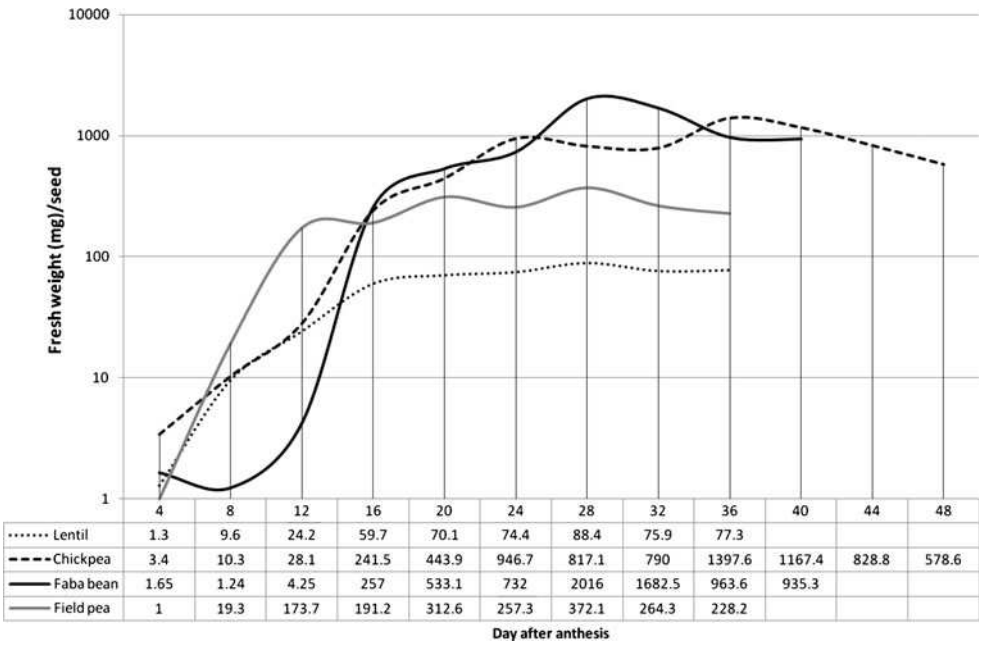
	Day 4		Day 8		Day 12		Day 16	
	Seed size (mm)	Embryo development	Seed size (mm)	Embryo development	Seed size (mm)	Embryo development	Seed size (mm)	Embryo development
Chickpea	2.5 ± 0.1	Globular	5.1 ± 0.1	Heart	9.5 ± 0.2	Early cotyledonary	5.7 ± 0.3	Cotyledonary
Lentil	1.0 ± 0.1	Pre-globular	2.7 ± 0.1	Globular to heart	5.0 ± 0.1	Heart to early cotyledonary	5.0 ± 0.0	Cotyledonary
Field pea	2.2 ± 0.1	Globular to heart	4.5 ± 0.1	Early cotyledonary	5.8 ± 0.2	Cotyledonary	8.9 ± 0.1	Cotyledonary
Faba bean	1.0 ± 0.0	Pre-globular	2.2 ± 0.1	Globular	1.6 ± 0.2	Globular to heart	4.0 ± 0.6	Early cotyledonary

Results

Seed physiological maturity, or when a seed reaches maximum dry weight, occurred approximately 8 days after peak fresh weight. Specifically, physiological maturity occurred at 36 DAA for lentil and field pea, 40 DAA for faba bean, and 48 DAA for chickpea (Fig. 1). Peaks in fresh weight occurred when concentrations of total ABA and its major precursors dropped, 36–40 DAA for chickpea, 28 DAA for lentil, 28 DAA for field pea, and 28–32 DAA for faba bean (Figs. 1, 2). The pattern formation to early maturation stage of embryo development was defined in each species (Fig. 3). Field pea was the fastest maturing legume with embryos at the heart shape stage by 4 DAA, early cotyledonary stage by 8 DAA, and cotyledonary stage by 12 DAA (Fig. 3). This is similar to previous embryo staging in *Pisum sativum* (Nadeau et al. 2011). Lentil and chickpea embryos were both at the heart shape stage 8 DAA and early cotyledonary stage by 12 DAA (Fig. 3) but chickpea took longer to reach maximum fresh weight (Fig. 1). Chickpea results are the same as those seen by Clarke et al. (2006). Faba bean was the slowest to develop, not reaching the heart shaped stage until 12 DAA and still in the early cotyledonary stage by 16 DAA (Fig. 3). Slight variability in the timing of embryo development for all legumes was observed. This may be due to environment, number of seeds per pod, or number of pods per peduncle (Table 2).

Cytokinin

In general, cytokinins were not a significant component of the seed hormone profile in any of the legume species examined. Only trace amounts of biologically active cytokinins (Z, dhZ, iP) were found from 4 to 12 DAA (Table 3). For chickpea, trace amounts of biologically



	4	8	12	16	20	24	28	32	36	40	44	48
Lentil	1.3	9.6	24.2	59.7	70.1	74.4	88.4	75.9	77.3			
Chickpea	3.4	10.3	28.1	241.5	443.9	946.7	817.1	790	1397.6	1167.4	828.8	578.6
Faba bean	1.65	1.24	4.25	257	533.1	732	2016	1682.5	963.6	935.3		
Field pea	1	19.3	173.7	191.2	312.6	257.3	372.1	264.3	228.2			

Fig. 1 Fresh weight (mg) per seed at 4–48 days after anthesis (DAA)

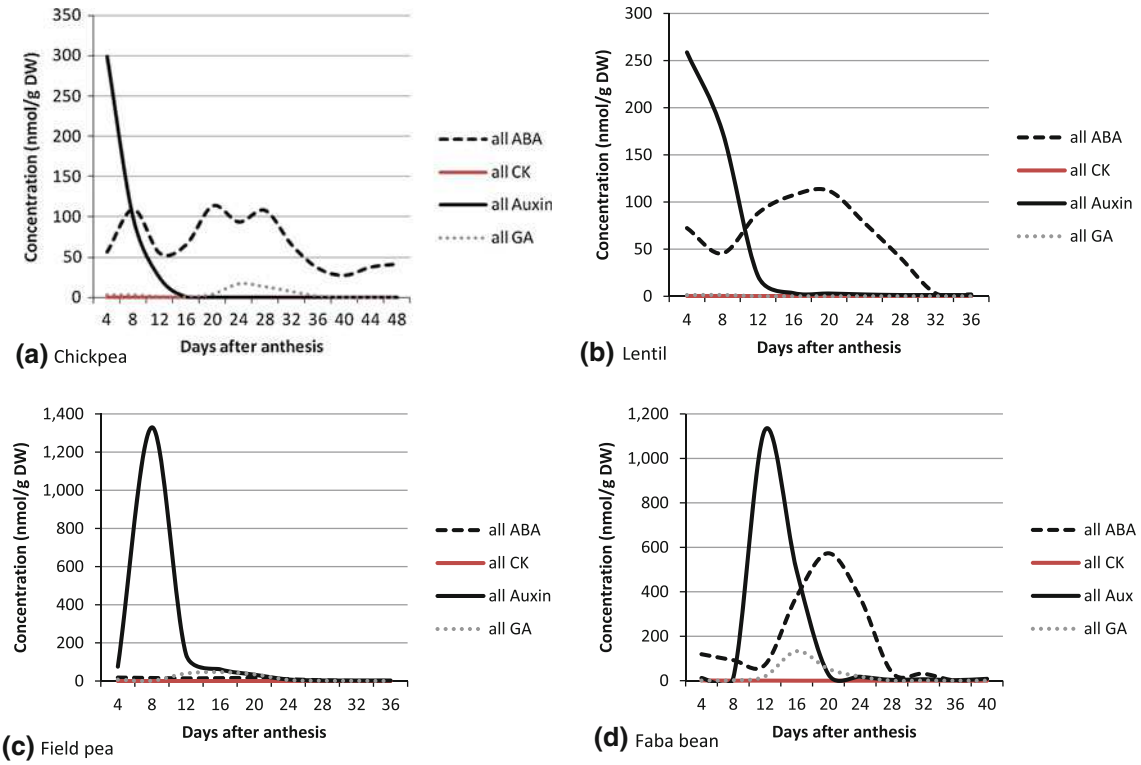


Fig. 2 Total hormone (active, derivatives, and precursors combined) concentration in nmol/g DW present at 4–48 days after anthesis in **a** chickpea (CDC Xena), **b** lentil (CDC Maxim), **c** field pea (CDC April), and **d** faba bean (Breeding line 219-18)

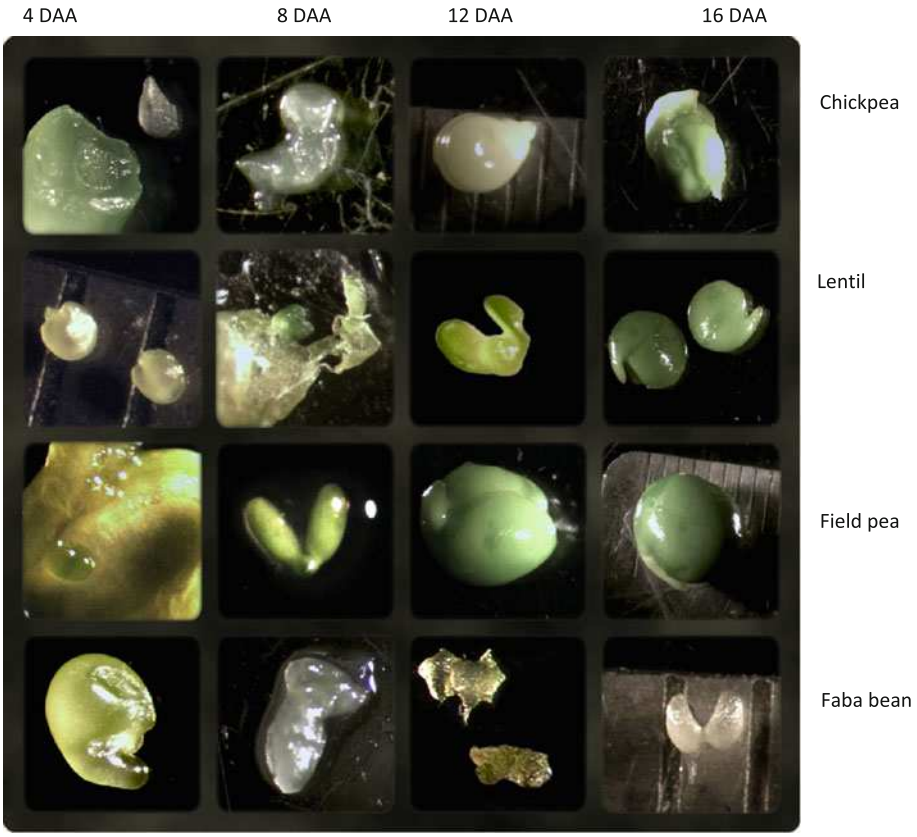


Fig. 3 Embryo development in chickpea (CDC Xena), lentil (CDC Maxim), field pea (CDC April), and faba bean (Breeding line 219-18) at 4–16 days after anthesis

Table 3 Cytokinins (nmol/g DW) present in seeds of chickpea (CDC Xena), lentil (CDC Maxim), field pea (CDC April), and faba bean (Breeding line 219-18) 4–48 days after anthesis (DAA)

DAA	Chickpea						Lentil					
	t-Z	t-ZR	c-ZR	dhZR	iP	iPR	dhZ	t-ZR	c-ZR	dhZR	iP	iPR
4	0.03	0.34	0.05	0.05	0.01	0.32	0.03	0	0.21	0.03	0.01	0.07
8	0.02	0.32	0.08	0.05	0	0.22	0	0	0.31	0	0	0.04
12	0	0.13	0.04	0.03	0	0.09	0	0.01	0.25	0	0	0.04
16	0	0.01	0.02	0	0	0.01	0	0	0.03	0	0	0.28
20	0	0	0	0	0	0	0	0	0	0	0	0.02
24	0	0	0	0	0	0	0	0	0	0	0	0
28	0	0.01	0	0	0.01	0.01	0	0	0	0	0	0
32	0	0	0	0	0	0	0	0	0	0	0	0
36	0	0	0	0	0	0	0	0	0	0	0	0
40	0	0	0	0	0	0						
44	0	0	0	0	0	0						
48	0	0	0	0	0	0						
LSD ($\alpha = 0.05$)	0	0.02	0.02	0.01	0.01	0.02	0.00	0.00	0.03	0.00	0.00	0.01

DAA	Field pea						Faba bean							
	t-Z	dhZ	c-ZR	dhZR	iP	iPR	t-ZOG	t-Z	dhZ	t-ZR	c-ZR	dhZR	iP	iPR
4	0.01	0.03	0.04	0.03	0.02	0.05	0	0	0.02	0.07	0.07	0.81	0	0.10
8	0	0.01	0.10	0	0	0.03	0	0	0.02	0.01	0.04	0.10	0	0.05
12	0	0.04	0.02	0	0	0.11	0	0.04	0.05	0.07	0.03	0.16	0	0.03
16	0	0	0.02	0	0	0.02	0	0	0	0	0.01	0	0	0.01
20	0	0	0	0	0	0	0.01	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0	0	0	0	0	0	0
32	0	0	0	0	0	0	0	0	0	0	0	0	0.03	0
36	0	0	0	0	0	0	0	0	0	0	0	0	0	0
40							0	0	0	0	0	0	0	0
LSD ($\alpha = 0.05$)	0	0.01	0.02	0	0	0.01	0.01	0.01	0.01	0.01	0.01	0.04	0.01	0.01

Bioactive cytokinins are highlighted. *dhZ* dihydro-zeatin, *dhZR* dihydro-zeatin riboside, *iP* isopentenyladenine, *iPR* isopentenyladenosine riboside, *Z* zeatin, *ZOG* zeatin-*O*-glucoside, *ZR* zeatin riboside

active cytokinins (Z and iP) were present at 4 and 8 DAA or from the globular to heart shape stage (Table 3); however, their biosynthetic precursors *cis*-ZR, dihydro-zeatin riboside (dhZR), and iPR were found in significantly higher amounts during this time period (Table 3). In lentil, trace amounts of biologically active cytokinins (dhZ and iP) were present at 4 DAA. However, the biosynthetic precursors *cis*-ZR and iPR were found in significantly higher amounts from 4 to 16 DAA or throughout the entire pattern formation stage (Table 3). For field pea and faba bean, trace amounts of biologically active cytokinins (Z, dhZ, and iP) were present at 4-12 DAA. Again, the precursors *cis*-ZR, dhZR, and iPR were found in significantly higher amounts from 4 to 12 DAA (Table 3). Faba bean contained the highest amount of biologically active cytokinins and the largest range of cytokinins with cytokinin precursors (t-ZR, c-ZR, dhZR, iPR) and the catabolism product t-ZOG. Lentils contained the highest percent of total cytokinin (from 4 to 12 DAA) to final seed weight at 25 % versus the standard of 4 % (Table 4).

Auxin

Auxin was the major hormone present during the pre-globular to globular stage of embryo development in four legumes (Fig. 2). Chickpea and lentil had similar total auxin profiles, with the auxin peak being on the downward slope 4 DAA (Fig. 2). Field pea and faba bean had similar total auxin profiles that showed a dramatic increase in auxin and auxin-amino acid conjugate formation at 4 DAA

Table 4 Percentage of total cytokinin measured from 4 to 12 DAA to final seed weight in grain legumes

Species	Total amount in nmol/g DW of cytokinin up to 12 DAA	Seed weight (g/100 seeds)	% Cytokinin to final seed weight
Chickpea	1.8	45	4
Lentil	1.0	4	25
Field pea	0.5	14	4
Faba bean	1.7	45	4

and 8 DAA, respectively (Fig. 2; Table 5). Field pea and faba bean contained 3–4 times the total auxin concentration of lentil and chickpea (Fig. 2). The auxin peak lasted from 8 to 12 days, after which it dropped and ABA became the main hormone present in the immature seeds for all species except field pea. At 8 DAA, IAA-Asp replaced IAA as the main auxin form in field pea and faba bean but IAA remained the predominant auxin form in lentil and chickpea (Table 5). 4-Cl-IAA was present in pea, lentil, and faba bean but not chickpea (Table 5). The peak concentration of 4-Cl-IAA was after the peak concentration of IAA and IAA-Asp in all species (Table 5).

In chickpea and lentil, auxin concentration was highest 4 DAA and was mainly represented by IAA (Table 5). In both species, IAA concentration dropped rapidly until 12 DAA. At this point, IAA-Asp concentration increased in chickpea whereas in lentil IAA-Asp concentration plateaued but remained the main auxin present from 16 to 36 DAA (Table 5). In lentil, 4-Cl-IAA peaked 8 DAA and was the main auxin present 12 DAA (Table 5). Indole-3-butyric acid (IBA) was uniquely present at 4–8 DAA in lentil (Table 5). In field pea and faba bean, total auxin peaked at 8 and 12 DAA, respectively, with the majority consisting of IAA, IAA-Asp, and IAA-Glu (Table 5). As IAA concentration dropped off rapidly, IAA-Asp remained the main auxin-conjugate until maturity (Table 5). 4-Cl-IAA peaked 12 DAA for field pea and 16 DAA for faba bean and was detected until 32 DAA (Table 5).

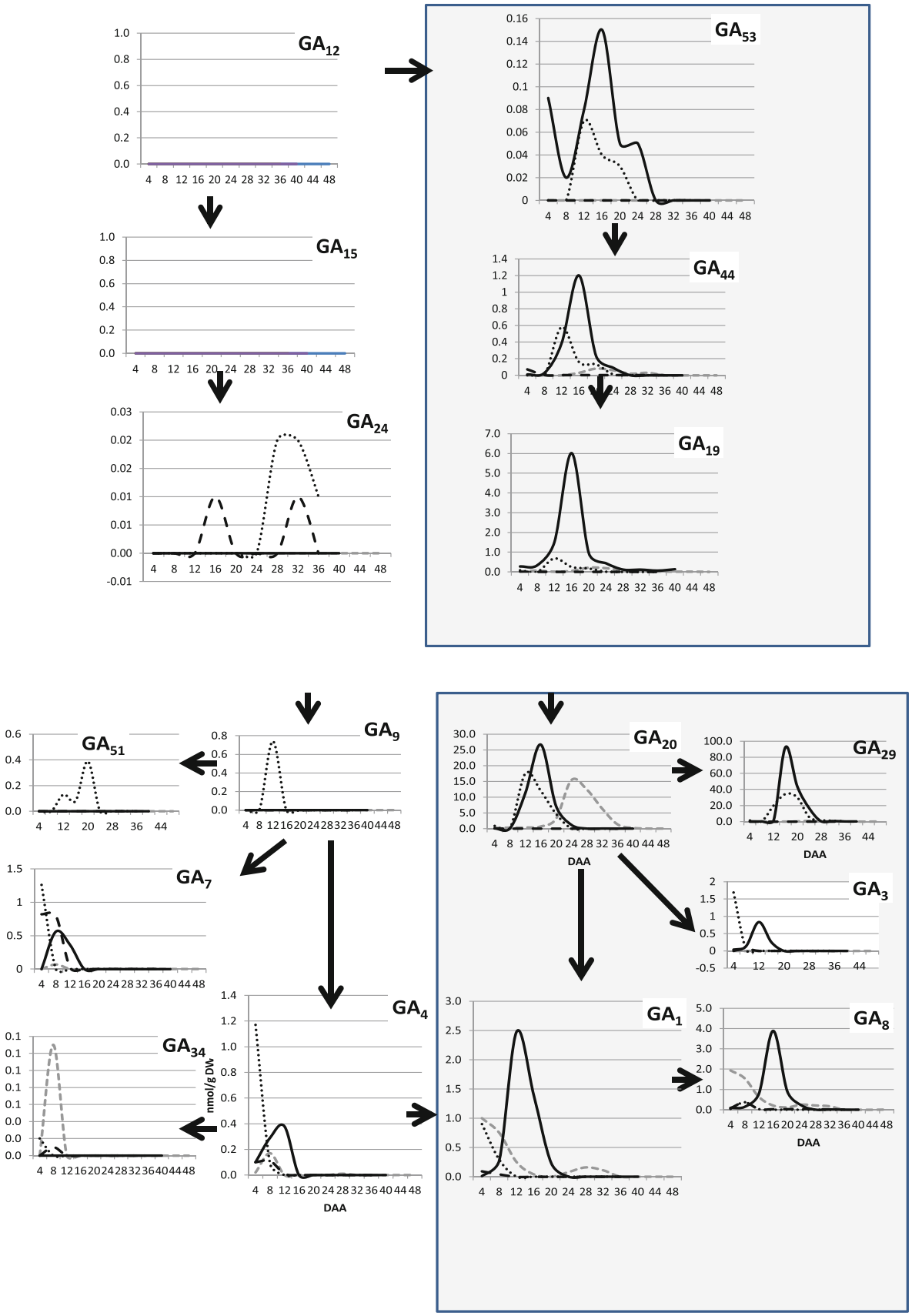
Gibberellin

Low levels of gibberellins were found in all species, with faba bean containing the highest concentration of total gibberellin (Fig. 2). In all species, low levels of polar (GA_1 and GA_3) and non-polar (GA_4, GA_7) bioactive gibberellins were present from 4 to 8 DAA. Twelve DAA, gibberellins were comprised primarily of the precursor or derivative forms from the 13-hydroxylation pathway, except for lentil (Fig. 4; supplemental Tables 1 and 2). In lentil, the non-13-hydroxylation pathway was

Table 5 Auxins (nmol/g DW) present in chickpea (CDC Xena), lentil (CDC Maxim), field pea (CDC April), and faba bean (Breeding line 219-18) at 4–48 days after anthesis (DAA)

DAA	Chickpea				Lentil					Field pea					Faba bean				
	IAA	IAA-Asp	IAA-Glu	IBA	IAA	IAA-Asp	IAA-Glu	IBA	4-Cl-IAA	IAA	IAA-Asp	IAA-Glu	IAA-Ala	4-Cl-IAA	IAA	IAA-Asp	IAA-Glu	IAA-Ala	4-Cl-IAA
4	295.35	3.91	0.07	0	233.89	22.39	0.50	1.41	0.49	57.68	17.52	0.32	0	0	10.47	1.58	0.04	0	0
8	93.02	4.31	0.17	0	137.33	15.09	0.61	3.77	17.63	355.73	919.95	42.19	0.53	11.18	4.48	7.45	0.18	0	0
12	15.89	8.44	0.04	0	6.67	3.21	0.01	0	11.47	3.61	118.54	2.52	0.03	15.58	507.88	602.6	14.71	0.18	2.3
16	0.48	0.61	0	0	0	2.79	0	0	0.47	1.08	51.76	0.67	0	7.87	119.15	353.6	6.57	0.02	15.0
20	0.32	0.23	0	0	0	2.82	0	0	0.04	0.24	29.78	0.31	0	3.01	14.98	8.47	0.27	0.01	5.0
24	0.14	0.05	0	0	0	1.80	0	0	0.07	0.18	7.79	0.10	0	0.19	7.20	9.38	0.21	0	1.1
28	0.14	0.01	0	0	0	1.27	0	0	0	0	3.04	0.06	0	0.01	0.72	2.79	0.12	0	0.2
32	0	0	0	0	0	1.27	0	0	0	0.05	2.61	0.09	0	0.04	0.52	5.52	0.05	0	0.3
36	0	0.01	0	0	0	1.20	0	0	0	0	2.84	0.13	0	0	0	2.26	0.00	0	0
40	0	0	0	0											0	8.30	0.05	0	0
44	0	0	0	0															
48	0	0.01	0	0.02															
LSD ($\alpha = 0.05$)	28.78	0.63	0.05	0.01	9.40	14.96	0.27	3.76	1.24	21.52	20.14	3.50	0.01	1.06	46.76	123.60	3.96	0.05	0.6

Known bioactive auxins are highlighted. *IAA* indole-3-acetic acid, *IAA-Ala N*-(indole-3-yl-acetyl)-alanine, *IAA-Asp N*-(indole-3-yl-acetyl)-aspartic acid, *IAA-Glu N*-(indole-3-yl-acetyl)-glutamic acid, *IAA-Leu N*-(indole-3-yl-acetyl)-leucine, *IBA* indole-3-butyric acid), *4-Cl-IAA* 4-chloroindole-3-acetic acid


GA12
GA15
GA24
GA53
GA44
GA19
GA9
GA51
GA7
GA34
GA4
GA20
GA29
GA3
GA1
GA8
nmol/g DW
DAA

◀**Fig. 4** Gibberellin biosynthesis in chickpea (*gray dashed line*), lentil (*black dashed line*), field pea (*dotted line*), and faba bean (*solid line*). The 13-hydroxylation pathway (polar gibberellins) is highlighted in *gray* and is the main pathway in all grain legumes except lentil. GA_1, GA_4, GA_7, and GA_3 are the bioactive gibberellin. Pathways are based on Yamaguchi (2008) and *graphs* indicate nmol/g DW at 4–48 DAA

of primary importance with GA_7 peaking 4–8 DAA. Unlike other species, lentil did not contain large amounts of gibberellin precursors at any time during seed development. Field pea appeared to have the most active gibberellin metabolism with the widest range of gibberellin forms (Fig. 4). Bioactive gibberellins peaked 4 DAA for field pea with an equal amount from both main pathways, but the highest amount of total gibberellins (both precursors and derivatives) was 12–16 DAA (Fig. 4).

Chickpea had two peaks in bioactive gibberellins—a main peak at 4 DAA and a second smaller peak around 28 DAA (Fig. 4 GA_1)—although its highest amount of total gibberellin was at 24 DAA (Fig. 2). Faba bean had the most gibberellin of all species with bioactive gibberellins, mostly GA_1, peaking 12 DAA (Fig. 4); its highest concentration of total gibberellins (mostly GA_{20} and its derivative, GA_{29}) was 16 DAA (Fig. 2).

ABA

Total ABA concentration increased with decreasing auxin concentration in all species and was the prominent hormone from early cotyledonary stage until maximum fresh weight in all species except field pea (Fig. 2). In all four legumes, the 8′-hydroxylation was the main ABA degradation pathway, leading to production of DPA (Figs. 5, 6; supplemental Tables 3 and 4). Two peaks in ABA concentration occurred in chickpea, at 8 DAA (heart shape stage) and 20 DAA (Fig. 5a). DPA then became the major ABA precursor throughout the rest of development and chickpea was the only legume where PA accumulated in significant concentrations (Fig. 5a). Lentil and faba bean had smooth total ABA concentration curves with peaks at 20 DAA (Fig. 2). In lentil, ABA peaked 12–16 DAA with 8′-hydroxylation pathway derivatives making up most of the total ABA after this time period (Fig. 5c). Field pea had a 5–10 times lower total ABA concentration than the other three legumes at peak production (Fig. 2), with the ABA apex occurring between 16 and 24 DAA (Fig. 6a). Moreover, ABA decreased at 24 DAA with no corresponding peak in DPA (Fig. 6a). Faba bean had six times the concentration

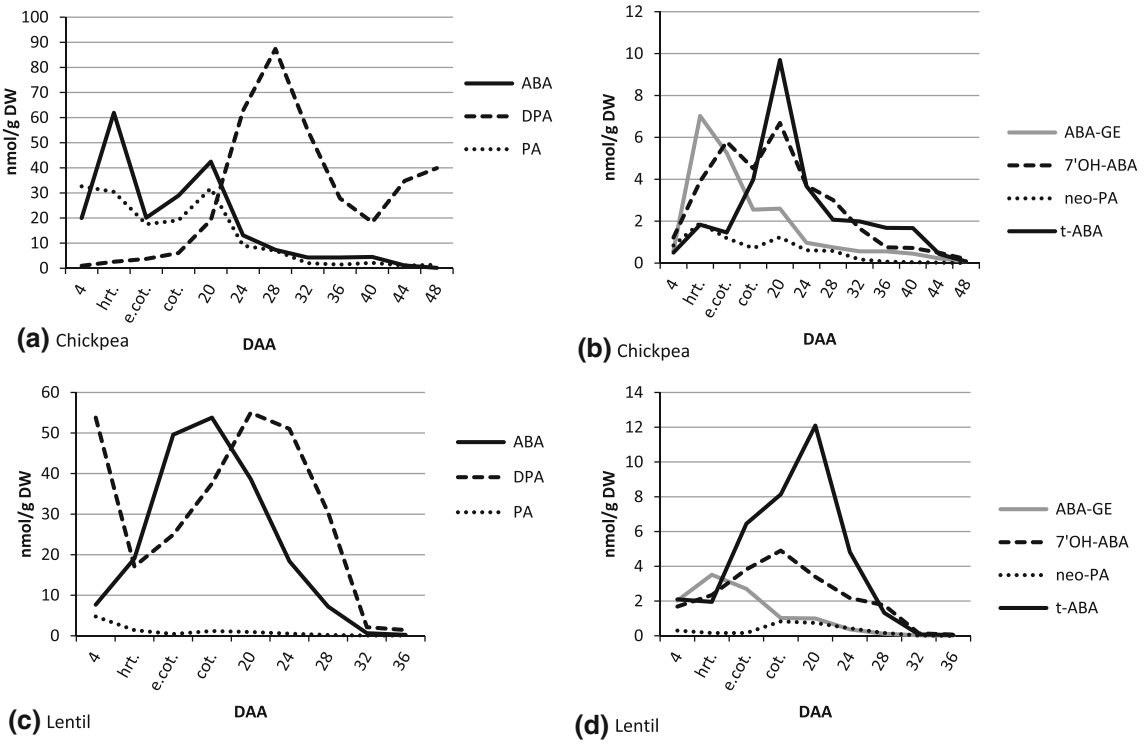


Fig. 5 ABA and precursors and derivatives (nmol/g DW) present in (**a** and **b**) chickpea (CDC Xena) and (**c** and **d**) lentil (CDC Maxim) at 4–48 days after anthesis (DAA). Embryo developmental stage is presented in place of DAA for 8 DAA at heart stage (hrt.), 12 DAA at early cotyledonary stage (e.cot.), and 16 DAA at cotyledonary stage (cot.)

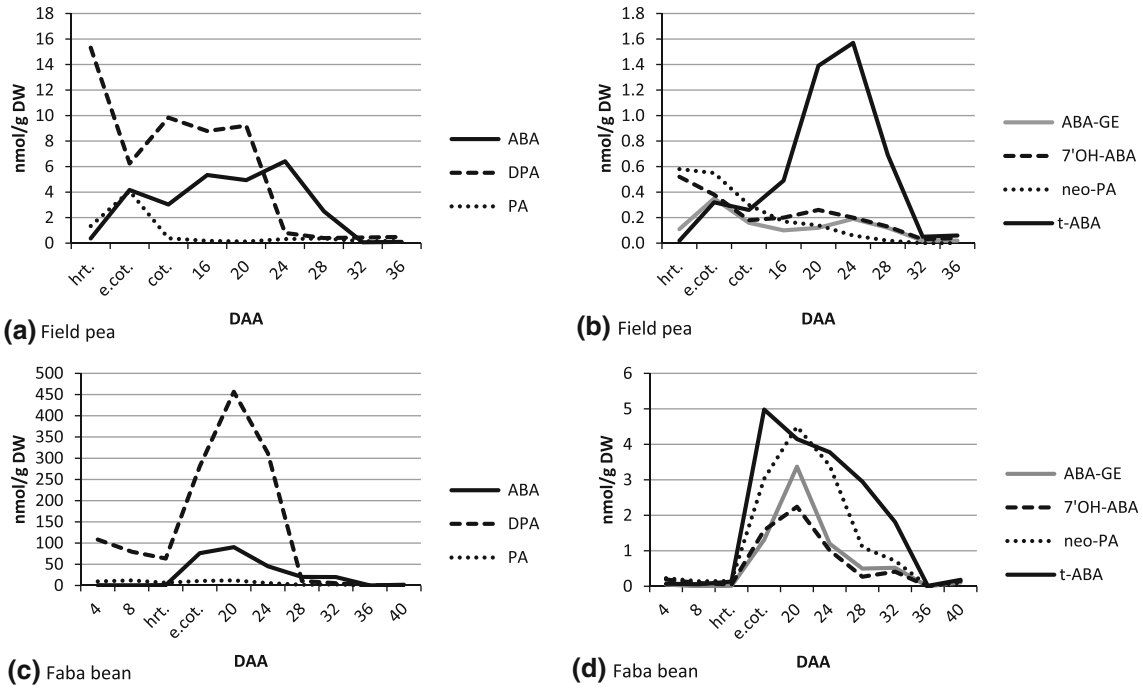


Fig. 6 ABA and precursors and derivatives (nmol/g DW) present in (**a** and **b**) field pea (CDC April) and (**c** and **d**) faba bean (Breeding line 219-18) at 4–40 days after anthesis (DAA). Embryo developmental stage is presented in place of DAA for heart shape stage (hrt.) at 4 DAA for field pea and 12 DAA for faba bean, for the early cotyledonary stage (e.cot.) at 8 DAA for field pea and 16 DAA for faba bean, and for the cotyledonary stage (cot.) at 12 DAA for field pea

of ABA and its precursors compared to chickpea and lentil (Fig. 2). Twenty DAA in faba bean, DPA and ABA concentrations peaked with DPA being four times greater than ABA (Fig. 6c). Except for faba bean, t-ABA concentration increased and then decreased with peak concentrations occurring at 4–8 days after the cotyledonary stage of development was achieved.

Discussion

Cytokinin and seed development

Our results confirm that cytokinins are predominantly found during the early pattern development phase, as per previous findings (Emery et al. 1998; Hwang et al. 2012; Miyawaki et al. 2004). The high concentration of cytokinin precursors versus bioactive cytokinins during early seed development is indicative of a high activity rate of these pathways rather than a lack of bioactive cytokinin. The iPR and iP pathway is consistently present in all four grain legumes (Table 3) and is the pathway linked to the female gametophyte in ferns (Menendez et al. 2009). The *cis*-ZR precursor was a major component of lentil and field pea, the *t*-ZR pathway was significant in chickpea, and the dhZR precursor pathway was more active in faba bean. Faba bean had a more active cytokinin profile in general, which may be linked to it having the largest seed (Table 1) and/or the slowest embryo development (Fig. 3). Based on the percentage of total cytokinin (up to 12 DAA) to final seed weight, lentil contained a significantly larger amount of cytokinin than all other grain legumes (25 versus 4 %) (Table 4). The reason for this is unknown but it may be due to lentil embryos developing from pre-globular to early cotyledonary stages from 4 to 12 DAA (Fig. 3) and having the smallest seed weight (Table 1). Previous research indicated that chickpea had a prominence of *cis*-isomers both during early pod set and later during grain filling (Emery et al. 1998). Our results indicate that the t-ZR pathway is more active during chickpea seed development than the c-ZR pathway, although this was variety dependent (Lulsdorf et al. 2013).

Cytokinin profiling stresses the importance of developmental context, where both the developmental time and space affect the cytokinin isomer produced. An example of this is the switch from the *cis*- to the *trans*-cytokinin form for maintenance of ovary viability in lupine (Emery et al. 2000). In *Arabidopsis*, the balance between these isomers reflects the activity of a plastid-localized versus cytosolic-localized pathway (Sakakibara 2006). In barley, increases in t-Z occur during grain fill, whereas c-Z is observed at the initial developmental stages (Powell et al. 2013). Finally,

in maize, ZR cytokinins are more predominant in the endosperm versus zeatin-9-glucoside in the embryo (Rijavec et al. 2011).

Auxin and seed development

IAA and its conjugates with aspartic and glutamic acids were the major auxins in the seeds of all four grain legume species studied. 4-Cl-IAA is a naturally occurring auxin in plants from the *Vicieae* tribe of the *Fabaceae* family (Engvild et al. 1981; Reinecke 1999) and was only found in the *Vicieae* tribe members (lentil, faba bean, and pea) (Fig. 5). Increasing 4-Cl-IAA concentrations are associated with decreasing IAA concentrations and, therefore, may be a degradation product (Table 5). Alternatively, 4-Cl-IAA may be a transportable seed factor involved in coordinating early pea seed and pericarp growth (Reinecke et al. 1995; Magnus et al. 1997), at least within the *Vicieae* tribe.

IAA-Asp concentrations increased after high IAA concentrations, indicating that IAA-Asp is a means of removing excessive amounts of free IAA and is a breakdown product of IAA (Ljung et al. 2002). However, the continuing high concentrations of IAA-Asp, without previous high IAA concentrations, make it the predominant auxin form during the pattern formation phase of field pea and faba bean and from the cotyledonary stage to physiological maturity in all the *Vicieae* species analyzed (Table 5). This, along with the specific mechanisms to ensure high concentrations of IAA-Asp in the developing seed (Ostrowski and Jakubowska 2011; Sasaki et al. 1994) and the fact that *Medicago* hydrolases can release IAA from IAA-Asp (Campanella et al. 2008), raises the question of whether IAA-Asp is an intermediate step in the catabolic pathway or a part of the dynamic auxin pool for seeds (Oetiker and Aeschbacher 1997; Rosquete et al. 2012). The similarity of field pea and faba bean auxin profiles and concentrations indicate that these species might spend a longer period of time in the cell division phase than chickpea or lentil, as opposed to being strictly phylogenetic in nature (Fig. 7). However, field pea develops faster and faba bean slower than either lentil or chickpea (Fig. 3). The reason for the high concentration and later peak of auxin in field pea and faba bean compared to lentil and chickpea is not known. Speculatively, these species have multiple seeds per fruit and their auxin profiles may be linked to fruit development. Auxin transport and fine concentration gradients across a few cells can affect plant development (Teale et al. 2006). In lentil, IBA is an important source of IAA in tightly regulated developmental contexts and is likely involved in maintaining fine IAA gradients in early seed development (Simon and Petrasek 2011). The reversible IAA amino conjugates, IAA-Leu and IAA-Ala, were not found or were in extremely low concentrations in all species (Table 5).

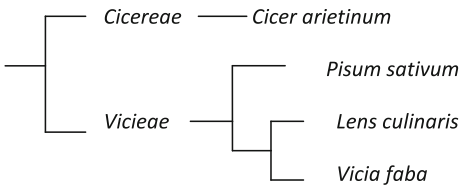


Fig. 7 Phylogeny of grain legumes (extracted from: Steele and Wojciechowski 2003)

Gibberellins and seed development

All four bioactive gibberellins (GA_1, GA_3, GA_4, and GA_7) were present in all four cool season legumes investigated. Although bioactive gibberellins were present mostly during the globular stage of embryo development, the precursors indicate that gibberellins peak just before ABA concentrations increase (Nadeau et al. 2011; Singh et al. 2010; Weber et al. 2005). Except for lentil, which used the non-13-hydroxylation pathway ($GA_{12} \rightarrow GA_{15} \rightarrow GA_{24} \rightarrow GA_9 \rightarrow \mathbf{GA_4} \rightarrow GA_{34}$) and, therefore, non-polar gibberellins, the other three legumes analyzed predominantly used the 13-hydroxylation pathway ($GA_{53} \rightarrow GA_{44} \rightarrow GA_{19} \rightarrow GA_{20}$ ($\rightarrow GA_{29}$) $\rightarrow \mathbf{GA_1} \rightarrow GA_8$) and polar gibberellins (Fig. 4). Despite this, the non-13-hydroxylation pathway was active during early seed development (4–8 DAA) in all these legumes (Fig. 4) and may be linked to tissue specific developmental patterns (Nadeau et al. 2011; Rodrigo et al. 1997). Chickpea was the only species that had two bioactive gibberellin peaks. Its double peaks of total gibberellin occurred at 4 and 24 DAA are 4 days before the total ABA peaks of 8 and 28 DAA. In chickpea, the ratio of total ABA and precursors to total gibberellin gave two peaks (Fig. 8); these occurred 16 and 44 DAA and do not appear to correlate with increased ABA or gibberellin concentrations. This highlights the fact that a single hormone concentration in and of itself is not enough to understand hormonal effects on seed development, but an analysis of the profile of all endogenous hormones is required for a complete picture.

ABA and seed development

ABA and/or its precursors (DPA, ABA-GE, PA, 7′-OH-ABA, *neo*-PA, and t-ABA) were detected throughout early seed development for all four cool season legumes. Lentil, faba bean, and pea (*Vicieae* species) had a single total ABA peak at approximately half way to physiological maturity (Fig. 2). This is similar to what was seen in soya bean (Liu et al. 2010). Specifically, ABA peaked at the early cotyledonary stage (Fig. 3) and continued until maximum fresh weight (Fig. 1). Chickpea had two ABA peaks, one at the heart shape stage (8 DAA) and one halfway through to physiological maturity (Fig. 5a). In *Arabidopsis*, two ABA

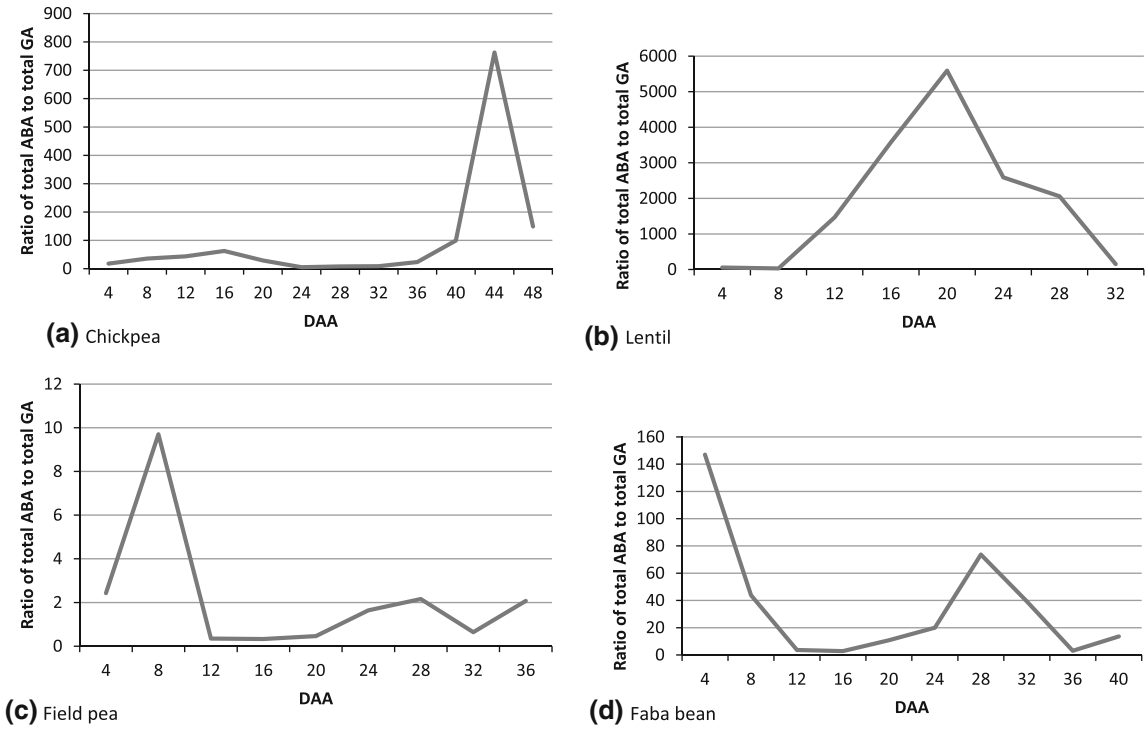


Fig. 8 Ratio of total ABA (active, derivatives, and precursors combined) and total GA at 4–48 DAA (days after anthesis) in **a** chickpea (CDC Xena), **b** lentil (CDC Maxim), **c** field pea (CDC April), and **d** faba bean (Breeding line 219-18)

peaks correspond to the middle stage of development [9 days after flowering (DAF)] and again towards the end of development (12 DAF) (Kanno et al. 2010). Kanno et al. (2010) report that the 9 DAF peak is mainly in the seed and is maternally produced ABA, whereas the 12 DAF peak is mainly in the siliques of Arabidopsis and is zygotically produced ABA. However, this system has flexibility when ABA deficient mutants are used, but zygotic ABA is required to achieve full seed dormancy (Kanno et al. 2010). Speculatively, the lack of two peaks in *Vicieae* species may be linked to maternal versus zygotic production or transport of ABA. Four pathways are reported to be involved in ABA catabolism (Nambara and Marion-Poll 2003). The high concentrations of DPA and PA noted here indicate that the 8′-hydroxylation pathway is the main oxidation pathway of ABA breakdown in legume species (Figs. 5, 6). Since chickpea is the only legume with significant concentrations of PA, the conversion of ABA to DPA is slowest in this species. Alternatively, since PA and 8′-hydroxy ABA interconvert and 8′-hydroxy ABA shows bioactivity, PA concentrations during early seed development may indicate the use of an alternative bioactive ABA in a specific developmental context (Jadhav et al. 2008). The lower total concentration of ABA in field pea compared to the other species is interesting, although there is no lack of dormancy associated with this genotype. Moreover, the lack of an increase in DPA following high ABA concentration (Fig. 5a) indicates that ABA is being converted to something that is not being measured. The increased concentration of ABA and its precursors in faba bean may be linked to seed size (Table 1). Despite the overall size of the peaks, the coordination of ABA and DPA synthesis, with DPA being produced in larger concentrations, indicates that the conversion of ABA to DPA is very fast relative to ABA synthesis and that only a small amount of active ABA is present. Finally, the photoisomerization of ABA to t-ABA is reversible and considered a non-biological process. However, the link between mid-cotyledonary stage embryos and the growth and decline of t-ABA concentration indicates that the production of t-ABA may be biologically relevant.

Since the maturation phase is distinguished by the ratio of ABA to gibberellin, we compared the ratio of total ABA and its precursors to total gibberellin and its precursors. The peaks were the same whether total ABA and precursors or just ABA was used. Chickpea gave two peaks at 16 and 44 DAA, lentil gave one peak at 20 DAA, field pea gave three peaks at 8, 28 and 36 DAA, and faba bean gave three peaks 4, 28, and 40 DAA (Fig. 8). Again, the similarities of field pea and faba bean as compared to chickpea and lentil with regard to number of peaks and relative ratios are interesting and may be linked to these species having multiple seeds per fruit (Table 1). Lentil is unique with respect to its relatively high ratio of total ABA to total GA,

being 6, 40, and 500 times the ratio found in chickpea, faba bean, and field pea, respectively (Fig. 8).

The overall aim of this research was to improve in vitro tissue and cellular culture with a focus on embryo rescue for rapid generation cycling protocols and improved medium for androgenic protocols or interspecific crosses. Assuming the first peak in total ABA to total gibberellin is the initiation of the maturation phase, the best time for embryo rescue is predicted to be around 16 DAA for chickpea, 16–20 DAA for lentil, 24–28 DAA for faba bean, and 16–24 DAA for field pea due to the lack of distinct spikes in ABA (Fig. 8). Because IAA-Asp concentration is high during the heart to early cotyledonary stage, especially in the multi-seeded fruit species (field pea and faba bean), the potential importance of IAA-Asp inclusion in legume androgenesis and interspecific embryo rescue protocols should be examined. Moreover, the usefulness of non-polar gibberellin during the promotion of embryo development up to the heart shaped stage should also be investigated, especially for lentil. In addition, the presence of low concentrations of ABA from the heart shape stage in chickpea and lentil is of interest; as is the unusual t-ABA synthesis curve at the end of the pattern formation stage in these species.

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