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**Androgenesis-inducing stress treatments change phytohormone levels
in anthers of three legume species (Fabaceae)**

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28 Abstract

29 Legumes are recalcitrant to androgenesis and only recently protocols were developed for pea (*Pisum sativum* L.) and
 30 chickpea (*Cicer arietinum* L.) albeit with low regeneration frequencies. Androgenesis is thought to be mediated
 31 through abscisic acid (ABA) but other phytohormones such as auxins, cytokinins and gibberellins have also been
 32 implicated. In view of improving protocols, hormone content of anthers was measured after exposure to cold,
 33 centrifugation, electroporation, sonication, osmotic shock or various combinations thereof using an analytical mass
 34 spectrometry-based analysis. Auxin had a key function during the induction process. High concentrations of IAA-
 35 Asp, a putative IAA metabolite, accumulated during the application of the different stresses in pea. In chickpea, the
 36 IAA-Asp concentration increased 30 fold compared to those of pea but only during the osmotic shock treatment and
 37 likely as a result of the presence of exogenous IAA in the medium. In contrast, no treatment showed such an
 38 increase in auxin content in the recalcitrant lentil (*Lens culinaris*). Of the various cytokinins monitored, only cZR
 39 increased after centrifugation and electroporation in pea and possibly chickpea. No bioactive gibberellins were
 40 detected indicating that this hormone group is probably not linked to androgenesis. In contrast to the other stresses,
 41 osmotic shock treatment caused a sharp reduction in the levels of all hormones analyzed, with the exception of
 42 auxins in chickpea. A short period of low hormone content might be a necessary transition phase required for
 43 androgenesis induction of legumes.

44 **KEYWORDS:** Absciscic acid, auxin, cytokinin, gibberellin, centrifugation, electroporation, sonication, osmotic
 45 shock

46

47 **Abbreviations:** ABA, Absciscic acid; ABA-GE, ABA-glucose ester; 7'OH-ABA, 7'-Hydroxy-ABA; BAP, Benzyl
 48 amino purine; C, Centrifugation; 4-Cl-IAA, 4-chloroindole-3-acetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid;
 49 DH, Doubled-haploid; DPA, Dihydrophaseic acid; E, Electroporation; GA₁₋₅₃, Gibberellins 1 – 53; IAA, Indole-3-
 50 acetic acid; IAA-Ala, N-(Indole-3-yl-acetyl)-alanine; IAA-Asp, N-(Indole-3-yl-acetyl)-aspartic acid; IAA-Glu, N-
 51 (Indole-3-yl-acetyl)-glutamic acid; IAA-Leu, N-(Indole-3-yl-acetyl)-leucine; IBA, Indole-3-butyric acid; 2iP,
 52 Isopentenyladenine; iPA, Isopentenyladenosine; NAA, 1-naphthaleneacetic acid; O, Osmotic shock; PA, Phaseic
 53 acid; S, Sonication; Z, Zeatin; ZOG, Zeatin-O-glucoside; dhZ, Dihydro-zeatin; ZR, Zeatin riboside

Introduction

Androgenesis is the development of plants containing only paternally derived chromosomes. Protocols for the development of double-haploid plants through androgenesis have been published for more than 200 species (Wedzony et al., 2009), but legumes are considered recalcitrant although recent breakthroughs have been made in the development of protocols for pea (*Pisum sativum* L.) and chickpea (*Cicer arietinum* L.) (Croser et al., 2006, Grewal et al., 2009, Ochatt et al., 2009). A soybean (*Glycine max* L. Merr.) androgenesis protocol has stalled at the embryo induction stage (Kaltchuk-Santos et al., 1997) and there is no protocol available for lentil (*Lens culinaris* Medik.).

In many species the induction of androgenesis requires the application of abiotic stresses such as heat, cold, or osmotic shock (Shariatpanahi et al., 2006). Cold stress is known to alter the division of the microspore nuclei from asymmetrical to symmetrical and this switch has been considered as one of the main routes in the formation of haploid cells and embryos (Rashid and Street, 1974; Smykal, 2000; Zaki and Dickinson, 1990). Cold treatment of plants or floral organs is thought to sustain microspore viability and to provide a period of carbohydrate starvation concomitant with an increase in the abscisic acid (ABA) levels (Žur et al., 2008). A 2 to 30 day cold stress of flower buds was important for induction of androgenesis in some French pea varieties, but more than 3 or 4 days of cold stress was detrimental in chickpea (Grewal et al., 2009, Ochatt et al., 2009). Osmotic stress is mediated through carbohydrates such as sucrose or maltose; they function as energy sources as well as osmotic regulators and influence both androgenic induction and embryo development (Ferrie et al., 1995; Hoekstra et al., 1993; Lionneton et al., 2001). Dunwell and Thurling (1985) observed a beneficial effect of a high level of sucrose on canola (*Brassica napus* L.) microspores and attributed it to the fact that a medium containing 17% sucrose generated an osmotic potential similar to that of an anther homogenate. A lower level of sucrose was subsequently required for a sustained level of microspore division (Ferrie et al., 1999; Lionneton et al. 2001). Similarly, carbohydrate requirements for pea and chickpea required a switch from 17% to a lower level of sucrose (Grewal et al., 2009; Ochatt et al., 2009).

The application of physical stressors such as centrifugation, electroporation, or sonication has been shown to improve the androgenic potential of various species. Centrifugation increased androgenesis induction of cultured anthers in *Datura innoxia* and in tobacco (*Nicotiana tabacum* L.) (Sangwan-Norreel, 1977; Tanaka, 1973).

Similarly, centrifugation showed a positive effect on androgenesis induction in chickpea and pea (Grewal et al., 2009; Ochatt et al., 2009). Electroporation appears to exert its effect through the introduction of medium components into the cells although a direct effect of electric pulses on androgenesis cannot be excluded since electric pulses were shown to stimulate protoplast growth and regeneration ability (Rech et al., 1987; Ochatt et al., 1988). Electroporated microspores developed embryos faster for *Ginkgo biloba* L. (Laurain et al., 1993), canola (Jardinaud et al., 1993), pea (Ochatt et al., 2009), and chickpea (Grewal et al., 2009). Similar to electroporation, sonication causes microcavity formation and collapse resulting in reversible permeability changes of cell membranes and thus facilitating the uptake of medium components (Fizzell, 1988; Joersbo and Brunstedt, 1992). Sonication using 40 KHz for 30 s enhanced androgenetic callusing in pea but only if combined with other stresses (Ribalta et al., 2010). The key for overcoming androgenetic recalcitrance in pea and chickpea was the pyramiding of multiple stressors including cold, osmotic shock, centrifugation, electroporation, and sonication (Grewal et al., 2009; Ochatt et al., 2009; Ribalta et al., 2010). The requirement of three or more stresses is unusual for androgenesis indicating that the mechanism in legumes is poorly understood.

Triggering embryo development in non-ovum cells is linked to stress, which in turn is linked to changes in endogenous hormone content. Absciscic acid is the 'stress' hormone since it plays a major role in adaptation of plants to adverse conditions (Kikuchi et al., 2006; Segui-Simarro and Nuez, 2008; Zavattieri et al., 2010; Žur et al., 2008). However, various other plant hormones are also affected by stress including auxins (IAA), cytokinins (zeatin), gibberellins (GA), or reactive oxygen species (Maraschin et al., 2005, 2006; Zavattieri et al., 2010). Cytokinin concentrations generally decrease in response to stress (Brugiere et al., 2003), whereas auxin responses vary (Jain and Khurana, 2009). Although gibberellins are not linked to abiotic stress, gibberellins prevent the stress response signal pathway being triggered (Gao et al., 2010). Interestingly, this pathway is down-regulated by heat and cold stress, but up-regulated by salt and drought stress indicating gibberellins may be effected by some stresses.

Given the need for effective androgenesis protocols in legumes and the evidence supporting the phytohormonal regulation of such a process, we have measured the concentrations of ABA, auxins, cytokinins and gibberellins in anthers of pea, chickpea, and the recalcitrant lentil after various stress treatments. We reasoned that ABA and likely auxin concentrations would increase as a result of the different stresses. Furthermore, we expected that pyramiding the different stresses would have an additive effect since only the combination of the different

108 stresses led to induction and regeneration. As a result of this investigation, we have established a baseline for the
 109 role of the four hormones classes during androgenesis induction of legumes but also show that osmotic shock
 110 treatment seems to have a different function compared to the other stresses.

111 **Materials & methods**

112 *Donor plant growth*

113 Plants were grown in the University of Saskatchewan greenhouses located in Saskatoon, Canada (52° 10.2'
 114 N, 106° 43.2 W). The regime consisted of 18 h light and 6 h dark with supplementation from high pressure sodium
 115 lamps. Temperatures were set to 22°C day/20°C night. Donor plants were grown in Sunshine® mix No. 4 with 5
 116 plants/11 L pot for chickpea and pea and Sunshine® mix No. 3 for lentil with 3 plants/ 8 L pot.

117 *Flower buds and anthers*

118 Flower clusters of chickpea containing different sizes of buds were picked from cultivar CDC Xena, from
 119 which buds ranging from 2.0 to 3.0 mm in length were selected for experiments (Grewal et al. 2009). Buds ranging
 120 in size from 6.0 to 8.0 mm in length were collected from pea (*Pisum sativum* L.) cultivars CDC April and Frisson
 121 (Ochatt et al. 2009). Flower buds from lentil (*Lens culinaris* Medic.) cultivar CDC Maxim were harvested when
 122 they were 1.5 to 2.0 mm in length. For cold treatments at 4°C, buds or clusters were kept in Petri® dishes on
 123 moistened filter paper. Microspores from all species were at the uni-nucleate stage (confirmed by DAPI staining).
 124 Approximately 500 mg of fresh weight was collected for each sample. All material was kept on ice during anther
 125 dissection and then freeze-dried. Buds required for the osmotic shock treatment were sterilized with 2% buffered
 126 bleach solution for 20 min, rinsed three times with sterile water, and anthers removed under sterile conditions
 127 (Grewal et al., 2009).

128 *Stress treatments*

129 Stress treatments for chickpea were applied according to Grewal et al. (2009) and according to Ochatt et al.
 130 (2009) for field pea except that the cold treatment of buds lasted 7 days (cold control) and the osmotic shock
 131 treatment was reduced from 7 to 3 days. For the control treatments, anthers were dissected out and immediately
 132 frozen at -80°C and later dried. For all other stress treatments, flower buds were kept at 4°C for 3 or 7 days and then

133 anthers were dissected out. Chickpea anthers were first transferred to a 0.4 cm electroporation cuvette filled with 1
 134 ml RM-IK17 (containing 22.83 μ M IAA and 1.86 μ M kinetin) and then either centrifuged (C) in an ICE Centra CL-
 135 2 centrifuge for 10 min at 168 g, or electroporated (E) with 1250 V/cm, 25 Ω and 25 μ F using three pulses, or kept
 136 in the same RM-IK17 medium for four days (O), or a combination thereof (C+O, E+O, C+E+O) (see Grewal et al.,
 137 2009 for details).

138 Regarding pea, anthers were transferred to a 0.2 cm cuvette with 1 ml HSO-17% sucrose, followed by
 139 centrifugation at 4°C (168 g for 10 min), or electroporation using 2500 V/cm, 50 Ω , and 100 μ F or sonication (S)
 140 with 42 KHz for 30 s (Branson Ultrasonic cleaners® Model 2510), or kept in osmotic shock medium (30 anthers in
 141 6 ml HSO-17% sucrose), or a combination thereof as described in Ochatt et al. (2009) and Ribalta et al. (2010).

142 Preliminary experiments showed that stress treatments similar to those of pea and chickpea were lethal for
 143 lentil microspore viability. Hence, only treatments that had confirmed microspore survival in preliminary
 144 experiments were selected for hormone profiling. After dissection, lentil anthers were either centrifuged at 168 g for
 145 3 min, or electroporated with 625 V/cm, 25 Ω , and 25 μ F, or treated with osmotic shock (500 anthers per plate with
 146 4.5 ml modified B5 medium plus 9% sucrose), or kept for 3 or 7 days in cold, or a combination thereof.

147 *Hormone analysis*

148 Quantification of ABA, cytokinins, auxins and GAs in control and stressed chickpea, field pea and lentil
 149 anthers was conducted at the Plant Biotechnology Institute of the National Research Council of Canada by UPLC-
 150 ESI-MS/MS (<http://www.nrc-cnrc.gc.ca/eng/facilities/pbi/plant-hormone.html>). The following hormones were
 151 quantified (1) ABA and ABA metabolites (*cis* and *trans* abscisic acid [ABA], phaseic acid [PA], dihydrophaseic
 152 acid [DPA], 7'-hydroxy ABA, *neo*-phaseic acid [*neo*PA], abscisic acid glucose ester [ABA-GE]; (2) auxins (indole-
 153 3-acetic acid [IAA], indole-3-butyric acid [IBA], indole-3-acetic acid aspartate [IAA-Asp], indole-3-acetic acid
 154 glutamate [IAA-Glu], N-(Indole-3-yl-acetyl)-alanine [IAA-Ala], N-(Indole-3-yl-acetyl)-leucine [IAA-Leu]); (3)
 155 cytokinins (*cis* and *trans* zeatin [Z], *cis* and *trans* zeatin riboside [ZR], *cis* and *trans* zeatin-O-glucoside [ZOG],
 156 dihydrozeatin [dhZ], dihydrozeatin riboside [dhZR], isopentenyl adenine [2iP], isopentenyl adenosine [iPA] and (4)
 157 gibberellins (GA1, 3, 4, 7, 8, 9, 19, 20, 24, 29, 34, 44, 51 and 53). Deuterated forms of the hormones were used as
 158 internal standards. They were either synthesized according to Abrams et al. (2003) and Zaharia et al. (2005) (d_3 -

DPA, d_5 -ABA-GE, d_3 -PA, d_4 -7'-OH-ABA, d_3 -*neo*PA, d_4 -ABA, d_4 -*trans*-ABA, d_3 -IAA-Leu, d_3 -IAA-Ala, d_3 -IAA-Asp, d_3 -IAA-Glu) or purchased from Cambridge Isotope Laboratories (Andover, MA, d_5 -IAA), Olchemim Ltd (Czech Republic, d_3 -dhZ, d_3 -dhZR, d_5 -Z-O-Glu, d_6 -iPA and d_6 -2iP) and Research School of Chemistry, Australian National University (Canberra, Australia, d_2 -GAs 1, 3, 4, 7, 8, 9, 19, 20, 24, 29, 34, 44, 51 and 53). The extraction and purification of samples was performed following an existing method described in Chiwocha et al. (2003, 2005) to which improvements were made (Han et al, unpublished).

Analysis was performed on a UPLC/ESI-MS/MS utilizing a Waters ACQUITY UPLC system, equipped with a binary solvent delivery manager and a sample manager coupled to a Waters Micromass Quattro Premier XE quadruple tandem mass spectrometer via a Z-spray interface. MassLynx™ and QuanLynx™ (Micromass, Manchester, UK) were used for data acquisition and data analysis. The procedure for quantification of multiple hormones has been described in detail by Chiwocha et al. (2003, 2005). Samples were injected onto an ACQUITY UPLC® HSS C18 SB column (2.1x100 mm, 1.8 μ m) with an in-line filter and separated by a gradient elution of water containing 0.02% formic acid against an increasing percentage of a mixture of acetonitrile and methanol (volume ratio: 50:50). Briefly, the analysis utilizes the Multiple Reaction Monitoring (MRM) function of the MassLynx v4.1 (Waters Inc) control software. The resulting chromatographic traces are quantified off-line by the QuanLynx v4.1 software (Waters Inc) wherein each trace is integrated and the resulting ratio of signals (non-deuterated/internal standard) is compared with a previously constructed calibration curve to yield the amount of analyte present. Calibration curves were generated from the MRM signals obtained from standard solutions based on the ratio of the chromatographic peak area for each analyte to that of the corresponding internal standard, as described by Ross et al. (2004). The QC samples, internal standard blanks and solvent blanks were also prepared and analyzed along each batch of tissue samples.

Statistical analysis

Experiments were replicated three times unless otherwise indicated; and means $\pm$ standard errors (SE) were calculated. Data reported in nM g⁻¹ dry weight (DW).

184 Results

185 Field pea (*Pisum sativum* L.) var. CDC April and Frisson

186 In CDC April and Frisson, the ABA concentration was highest in control anthers with 2.1 and 2.6 nM g⁻¹
 187 DW, respectively. All stress treatments reduced ABA content (Table 1). Centrifugation increased t-ABA levels in
 188 CDC April whereas in Frisson, this isomer increased after cold and combinations of cold with electroporation,
 189 centrifugation, or sonication. The presence of high levels of DPA and PA in both species indicates that a rapid
 190 breakdown of ABA is taking place via the 8'-hydroxylation pathway. For CDC April, DPA spiked at 131.0 nM g⁻¹
 191 DW when no stress was applied (control) whereas PA peaked after cold stress with 173.2 nM g⁻¹ DW. In Frisson,
 192 the highest PA level was measured in control anthers with 189.1 nM g⁻¹ DW and the highest DPA level of 51.2 nM
 193 g⁻¹ DW in the cold plus centrifugation treatment. *Neo*-PA levels were highest in the control treatment of both
 194 varieties but levels indicate that they played only a minor role. The values for 7'-OH-ABA and ABA-GE were near
 195 detection limits and thus were of little significance to the androgenesis process. However, the treatments involving
 196 osmotic shock differed from the other treatments since ABA and its catabolites attained very low values especially
 197 in the combination of cold +C+E+S+O (Table 1).

198 Regarding auxin, IBA was not detected in any of the three species. The IAA concentration of CDC April
 199 was 1.6 nM g⁻¹ DW for both control and cold-treated anthers (Table 2). This concentration increased about two fold
 200 after the cold plus centrifugation treatment. The other stress treatments only slightly raised IAA levels compared to
 201 the control. IAA-Asp content peaked after application of all stresses except osmotic shock with the highest value of
 202 42.7 nM g⁻¹ DW attained in the cold+C+E treatment. In contrast, combining the different stresses with osmotic
 203 shock treatment (cold+E+C+S+O) led to very low levels of IAA and its catabolites (Table 2). IAA-Glu, another
 204 auxin catabolite, was only detected in small amounts whereas the reversible IAA storage forms, IAA-Ala and IAA-
 205 Leu, were either not present or only detected in minute amounts close to detection levels. The IAA concentration of
 206 var. Frisson was generally lower than in CDC April (Table 2). Compared to control anthers, centrifugation only
 207 doubled the IAA concentration in this cultivar; it did not quadruple it as in CDC April indicating that the two
 208 genotypes respond somewhat differently to this stress. In contrast, cold plus sonication was a more effective stress
 209 treatment in Frisson which increased the IAA concentration to 2.8 nM g⁻¹ DW although this concentration was not
 210 significantly different from the cold +C or cold +E treatments. IAA-Leu and IAA-Ala were not detected in these

samples. Frisson also displayed very high concentrations of IAA-Asp of up to 45.7 nM g⁻¹ DW as a result of the various stress treatments except those involving osmotic shock (Table 2). Similar to CDC April, the latter treatment again reduced the content of auxin and its catabolites to low levels.

The free base bioactive cytokinins tZ, cZ, or dhZ, were either not detected or present in very low concentrations in anthers of both varieties. The major forms were cZR, iPA as well as 2-iP (Table 3). The cis isomer of ZR spiked in the centrifugation treatment of CDC April with 1.8 nM g⁻¹ DW. Compared to the control, cZR was significantly higher after application of stress in Frisson but the difference among the stress treatments was minor. In CDC April, the 2iP concentrations were lower in the stress treatments (0.5 - 0.7 nM g⁻¹ DW) compared to the control (0.9 nM g⁻¹ DW) but similar or slightly higher for Frisson. The osmotic shock treatment again either completely removed or reduced the different cytokinin forms to very low levels in both varieties (Table 3).

No bioactive gibberellins (GA₁, GA₃, GA₄ and GA₇) were detected in pea anthers of both varieties, only precursors (GA₉, GA₁₉, GA₂₀, GA₂₄, GA₃₄, GA₄₄ and GA₅₃) and catabolites (GA₂₉, and GA₅₁). A notable genotype difference between the two field pea cultivars tested was that CDC April had GA precursors for both the non-13-hydroxylation pathway (GA₉ and GA₂₄ are precursors for GA₄) and the early-13-hydroxylation pathway (GA₁), whereas Frisson only contained precursors related to the latter pathway. Control anthers contained the widest variety of gibberellins (Table 4). After the various stress treatments, GA₁₉ was the major component in both varieties with concentrations of 2.0 nM g⁻¹ DW in the cold +C+E treatment of CDC April and 1.9 nM g⁻¹ DW in the cold treatment of Frisson. Similar to the other hormones, the osmotic shock treatment reduced the concentration of this hormone group to zero or to minute amounts of GA₁₉ (Table 4).

Chickpea (*Cicer arietinum* L.) var. CDC Xena

The ABA content of control anthers (10.4 nM g⁻¹ DW) was significantly higher than after the different stress treatments. Application of a 3-day cold treatment to flower buds reduced this concentration by about 50% (Table 1). Both centrifugation and electroporation further reduced ABA content. Compared to field pea, DPA levels of cold-treated anthers were much lower in chickpea and reached only a maximum of 6.3 nM g⁻¹ DW. PA concentrations in control and cold-treated anthers were also lower with maximum values of 26.1 and 21.3 nM g⁻¹

237 DW, respectively. However, the combination of cold with either centrifugation or electroporation resulted in very
 238 high PA levels of more than 100 nM and moderate DPA concentrations (Table 1). The various combinations with
 239 osmotic shock reduced ABA and its metabolites to very low levels (Table 1).

240 Regarding auxins, control anthers contained on average only 0.6 nM g⁻¹ DW IAA as well as 0.07 nM g⁻¹
 241 DW of IAA-Asp. The IAA storage forms, IAA-Ala and IAA-Leu, as well as the conjugate IAA-Glu were not
 242 detected (Table 2). Three days of cold treatment of buds slightly reduced the auxin content. In contrast to field pea,
 243 the medium used during centrifugation, electroporation and osmotic shock treatments contained 22.83 µM IAA.
 244 Since the effect of the centrifugation treatment is thought to be mediated through an increase in the auxin content,
 245 this was tested by analyzing anthers which had been centrifuged either with or without IAA in the medium. Data
 246 shows that the cold plus centrifugation treatment without IAA (0.43 nM g⁻¹ DW) was not different from the cold
 247 control indicating that centrifugation itself does not increase auxin content of chickpea anthers. Adding IAA to the
 248 medium during centrifugation increased the concentration about 13 times to 5.57 nM g⁻¹ DW. Similarly, adding IAA
 249 during the electroporation treatment also led to an increased IAA concentration (3.37 nM g⁻¹ DW) compared to
 250 electroporation without IAA (0.14 nM g⁻¹ DW). However, it should be noted that these data were not replicated.
 251 Even though these stresses increased IAA levels, the IAA-Asp levels only increased slightly likely due to the short
 252 exposure time. If after the cold treatment, the anthers were kept on osmotic shock medium for four days, the IAA
 253 concentration increased due to the presence of the exogenous IAA. However, the IAA-Asp concentrations showed
 254 extremely large spikes with more than 1,000 nM g⁻¹ DW. This amount is about 30 times more than that measured in
 255 pea and some of this excess auxin was stored in form of IAA-Ala in an accessible form or further metabolized to
 256 IAA-Glu.

257 The major bioactive forms of cytokinin detected in chickpea control anthers were tZR, cZR and iPA with
 258 levels of 1.2, 0.8, and 0.4 nM g⁻¹ DW, respectively. Only cZR increased after cold stress albeit not significantly
 259 (Table 3). The medium contained 1.86 µM kinetin during the centrifugation, electroporation, and osmotic shock
 260 treatments. Centrifugation and electroporation of anthers led to a temporary increase in cZR (2.9 and 3.1 nM g⁻¹ DW
 261 and iPA (0.9 nM g⁻¹ DW) but not in tZR (0.6 and 0.5 nM g⁻¹ DW). In contrast, cytokinin content of anthers exposed
 262 to osmotic stress declined significantly; tZ, tZR, and dhZ were no longer detected and cZR, 2iP, iPA and dhZR
 263 levels were very low even though the exposure to kinetin was over a 4-day period (Table 3).

Similar to field pea, none of the biologically active forms of gibberellin were detected in chickpea in control anthers or after any of the stress treatments (Table 4). However, the GA₁ precursors GA₅₃, GA₂₀, GA₁₉, and GA₉ of the 13-hydroxylation pathway were present as well as the catabolite GA₂₉. GA content peaked in control anthers with 1.8 nM GA₈ and 2.6 nM g⁻¹ DW GA₂₀ whereas GA₁₉ and GA₂₉ increased slightly after the cold treatment. The osmotic stress treatment completely eliminated this hormone with the exception of GA₁₉ which was still present in very small amounts (Table 4).

Lentil (*Lens culinaris* Medik.) var. CDC Maxim

Lentil treatments were cold storage of buds for 3 or 7 days, osmotic shock of anthers for 3 days at 4°C, electroporation of anthers followed by osmotic shock for 3 days at 4°C, and centrifugation of anthers followed by osmotic shock for 3 days at 4°C. No growth regulators were applied during stress treatments. Control anthers contained 1.1 nM g⁻¹DW ABA. The lowest ABA concentration was observed in the 7d cold treatment with 0.4 nM g⁻¹ DW and the highest level of more than 1.3 nM g⁻¹ DW after the osmotic shock treatments (Table 1). This is in contrast to pea and chickpea where osmotic shock resulted in the lowest ABA levels. Control anthers contained high levels of PA (182.6 nM g⁻¹ DW) similar to pea var. Frisson. The 3-day cold treatment about halved this amount but the lowest levels were achieved in the 7-day cold treatment. DPA concentrations peaked in anthers from the 3-day cold treatment followed by the control. However, the osmotic shock treatments in conjunction with cold could not reduce ABA or PA and DPA concentrations to the levels in the other two pulses (Table 1).

The IAA content of control anthers was 1.0 nM g⁻¹ DW and increased to 3.7 nM after the 7-day cold treatment (Table 2). This concentration was higher than the IAA content for var. Frisson but lower than the maximum value for the cold +C treatment of var. CDC April. The major difference between lentil and the other two pulses was in the IAA-Asp content. IAA-Asp only reached a maximum concentration of 1.3 nM g⁻¹ DW in the control and the 7-day cold treatment (Table 2) whereas the other species achieved much higher values of more than 40 nM for pea and 1,200 nM g⁻¹ DW for chickpea.

Similar to pea and chickpea, the major cytokinins in lentil anthers were cZR, 2iP, and iPA with the highest concentrations in the 3-day cold treatment and in the control (Table 3). The osmotic shock treatments

290 reduced cytokinin content to a level similar to that of pea and chickpea.

291 Eight different kinds of gibberellins were detected in lentil anthers but similar to pea and chickpea, no
 292 bioactive forms were found (Table 4). The control anthers contained the highest amounts of GA₂₉ and GA₈ which
 293 are GA₁ catabolites. GA₄₄ and GA₁₉, both precursors of GA₁, were also detected. The 7d cold and the osmotic shock
 294 treatments reduced GA content to low levels (Table 4) similar to pea and chickpea.

295 Androgenesis inducing stress treatments caused an increase in the proportional levels of active auxins as
 296 compared to all other hormones measured in all species tested. Specifically, osmotic shock is causing the
 297 proportional increase in IAA for Frisson, while it required a pyramiding of stress treatments and osmotic shock to
 298 achieve the same proportions in CDC April and CDC Xena (data not shown). This holds true for CDC Maxim
 299 except the total amount of proportional difference is 2-3 times lower and it appears to be caused by 7 days at 4°C.
 300 CDC April has a greater amount of IAA/ABA than all other pulses tested when pyramiding treatments. Although
 301 the spike in active auxin to ABA levels is the greatest in CDC April, this trend is seen in all other genotypes and
 302 indicates that auxin may be more important than ABA for triggering androgenesis in legumes.

303

304 Discussion

305 Our investigation showed that androgenesis in legumes is mediated via phytohormones and that auxin
 306 played a major role in this process after application of different stresses. Auxin is involved in many plant growth
 307 functions including flowering and fertilization (Aloni et al., 2006; Hirano et al., 2008; Tromas et al., 2010; Zhao,
 308 2010). In pea, IAA levels spiked after centrifugation in var. CDC April to about five times the concentration of the
 309 control anthers (Table 2). In var. Frisson, IAA levels merely doubled in all stress treatments compared to the control
 310 with a slightly higher response after sonication. However, osmotic shock in combination with the other treatments
 311 (cold+C+E+S+O) reduced IAA levels considerably. In chickpea, IAA levels increased after centrifugation,
 312 electroporation, and osmotic shock compare to the control or cold treatment but this was likely the result of the
 313 exogenous IAA in the medium. In lentil, the IAA concentration after 7 days of cold was similar to levels in pea var.
 314 Frisson after cold plus sonication (Table 2). Tanaka (1973) was the first to report that centrifugation of tobacco
 315 anthers in the presence of IAA improved androgenesis induction. In contrast, Sangwan-Norreel (1977) found a

positive effect of centrifugation without the addition of hormones to *Datura innoxia* anthers. The requirement for exogenous auxin during centrifugation seems to be species-dependent.

The major hormone in the two responsive legumes was the IAA conjugate, IAA-Asp. Anthers of both pea and especially chickpea contained extremely high concentrations of IAA-Asp after cold, centrifugation, electroporation or sonication stress in pea of up to 46 nM g⁻¹ DW or after osmotic shock in chickpea of up to 1,284 nM g⁻¹ DW (Table 2). In contrast, the recalcitrant lentil had no such peaks and the maximum concentration of IAA-Asp was less than 1.3 nM g⁻¹ DW (Table 2). This huge concentration difference might be related to the lack of response of lentil to androgenesis induction. Whether the increase in IAA-Asp seen in microspores of legumes is due to an increased breakdown of IAA or whether it is due to an increased need for IAA-Asp for some unknown function is unclear. Most authors consider IAA-Asp a degradation product of IAA and if there is an increase in the metabolites going through the tryptophan-dependent pathway, there will be a corresponding increase in IAA degradation products (Normanly et al., 1995). Potentially, the various stresses applied to pea and chickpea caused IAA spikes which quickly degrade causing IAA-Asp spikes. In contrast, several authors have suggested that IAA-Asp may be biologically active and its function more complex than merely a mechanism for removal of free IAA (Böttcher et al., 2010; Oetiker and Aeschbacher, 1997). The GH3 protein catalyzes the conjugation of IAA to IAA-Asp. GH3-mediated auxin homeostasis may have a role in a plant's response to stress and be necessary for re-allocation of resources for survival (Ghanashyam and Jain 2009, Park et al., 2007; Simon and Petrášek, 2011). Ostrowski and Jakubowska (2011) found this enzyme to be most active in the early stages of pea seed development (5 mm stage), implying a potential need for IAA-Asp. High concentrations of IAA-Asp (3,231 pM g⁻¹ FW) have been found in mature anthers of rice (Hirano et al., 2008), connected to ripening in grape and tomato and have been linked to temperature sensitivity in cell cultures of henbane (Böttcher et al., 2010; Oetiker and Aeschbacher, 1997). Similarly, Sasaki et al. (1994) showed that embryogenic cells of carrot not only have 15 times more IAA, but also an increase in IAA-Asp, whereas the non-embryogenic cells had Ox-IAA-Asp (oxindole-3-acetylaspartic acid), a potential degradation product of IAA-Asp.

The chlorinated auxin, 4-Cl-IAA, could not be investigated in this analysis and IAA-Asp production in field pea could also be linked to this potent hormone which is present in pea fruits with a unique mode of action (Engvild et al., 1981; Park et al., 2010). Its biosynthesis is independent of IAA and has a higher activity in pea,

343 which may be linked to increased stability (DeMason and Polowick, 2009, Simon and Petrásek, 2011). 4-Cl-IAA
344 may also be present in lentil but has not been reported in chickpea (Engvild et al., 1981; Reinecke, 1999).

345 The concentration of ABA and its catabolites spiked in control and cold-treated anthers and decreased after
346 osmotic shock treatment in the three legume species investigated (Table 1). Similarly, cold pre-treatment was found
347 beneficial for barley (*Hordeum vulgare* L.), tobacco (*Nicotiana tabacum* L) or triticale (*xTriticosecale*) androgenesis
348 due to improved microspore viability and increased response (Imamura and Harada, 1980; van Bergen et al., 1999,
349 Wang et al., 1999, Žur et al. 2008). However, pyramiding the other stresses such as centrifugation or electroporation
350 on top of the cold stress did not increase ABA levels indicating that there was no additive effect from the different
351 treatments. In contrast, osmotic shock treatment reduced ABA and catabolite content to very low levels especially in
352 the combined treatment cold +C+E+S+O in pea and cold +C+E+O in chickpea.

353 The predominant cytokinin was cZR in all three legumes but chickpea also responded with higher iPA
354 concentrations whereas both pea varieties showed increases in 2iP after some stresses (Table 3). Reports on
355 embryogenesis found that the *cis*-isomers predominated during pea and chickpea seed development especially
356 during the heart-shaped embryo stage (Emery et al., 1998; Quesnelle and Emery, 2007). No information is available
357 on the active cytokinin forms in lentil. Trans-isomers are thought to be biologically more active (Sakakibara, 2006;
358 Werner and Schmülling, 2009). However, Quesnelle and Emery (2007) showed that pea embryos could grow
359 equally well with either *cis*- or *trans*- zeatin indicating that cZ or its riboside are active cytokinins in this species. In
360 pea and chickpea, the highest cZR concentration occurred after application of the centrifugation and electroporation
361 stresses but in control and 3d cold-treated lentil anthers. Tret'yakova et al. (2009) found that the total cytokinin
362 content increased almost two fold from 284 to 1,324 ng g⁻¹ DW in androgenetic callus of larch (*Larix sibirica*
363 Ledeb.). In our experiments, osmotic shock treatment reduced cytokinin content to very low levels (Table 3). Sáenz
364 et al. (2010) reported that the total cytokinin content in coconut (*Cocos nucifera* L.) was lower in embryogenic
365 compare to non-embryogenic callus. Hence, it seems that the effect of the osmotic shock treatment in reducing
366 hormone content might be of significance in the induction process. Exogenously supplied kinetin was present during
367 the 4-day osmotic shock treatment of chickpea anthers but this did not result in increased cytokinin levels (Table 3)
368 even though this hormone is rapidly metabolized (Sakakibara, 2006).

369 Although gibberellins are required for normal pollen, anther and seed development, a complete

understanding of their specific function remains unknown (Swain and Singh, 2005). In our study, no bioactive GAs were found not even in the control anthers (Table 4). It is feasible that we collected anthers at a stage when GA content started to drop off or that there was a dilution effect of the anther cells on the measurement of GA levels in the microspores. However, it is more likely that we interrupted the formation of GAs, hence the increase in precursors, by triggering stress reactions. Despite the need for this hormone immediately after fertilization (Alabadi et al., 2009; Dorcey et al., 2009), GAs suppress the embryogenic LEC transcription factor cascade which initiates embryogenesis. It is not surprising that stresses which trigger androgenesis would decrease its presence. A plant's gibberellin levels are particularly controlled by the biosynthesis enzymes GA₂₀-oxidase and GA₃-oxidase and the catabolism enzyme GA₂₀-oxidase (Fleet and Sun, 2005). It could be that the stress treatments which induce androgenesis were suppressing the conversion of GA₁₉ to GA₂₀ or the production of the GA₂₀-oxidase enzyme. However, this enzyme is also important in the formation of GA₁₉ from GA₅₃ and GA₄₄. A similar effect was reported in wheat tissues where the GA₁₉ intermediate had an especially low affinity for the GA₂₀-oxidase enzyme and therefore tended to accumulate (Appleford et al., 2006).

Osmotic shock treatment seems to be different from the other stresses because this treatment reduced the content of all hormones with the exception of IAA and IAA-Asp in chickpea. The high concentration of IAA-Glu in chickpea indicates that the IAA concentration in the medium could be too high or the exposure to this hormone too long. Since a short period of low hormone content seems to be a necessary transition phase required for the switch from pollen development to embryo induction in pea, it would be interesting to determine if a reduced IAA concentration in the medium or a secondary period of osmotic shock treatment without IAA would improve induction in this species.

Androgenesis induction was successful for pea and chickpea but not for lentil. Our data showed that this might be linked to auxin with a possible involvement of IAA-Asp. The ratio between auxin and ABA also indicates that androgenesis in legumes has a greater auxin component than other species especially cereals, where increased androgenesis and somatic embryogenesis was related to increased ABA levels (Wang et al., 1999; Žur et al., 2008). We are clearly only at the beginning of understanding what governs androgenesis induction in legumes.

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399 References

- 400 Abrams SR, Nelson K, Ambrose SJ (2003) Deuterated abscisic acid analogs for mass spectrometry and metabolism
 401 studies. *J. Labelled Compd. Radiopharmaceut.* 46:273-283
- 402 Alabadi D, Blázquez MA, Carbonell J, Ferrándiz C, Pérez-Amador MA (2009) Instructive roles for hormones in
 403 plant development. *Int J Dev Biol* 53:1597-1608
- 404 Aloni R, Aloni E, Langhans M, Ullrich CI (2006) Role of auxin in regulating *Arabidopsis* flower development. *Planta*
 405 223:315-328
- 406 Appleford NEJ, Evans DJ, Lenton PG, Croker SJ, Devos KM, Phillips AL and Hedden P (2006) Function and
 407 transcript analysis of gibberellin-biosynthetic enzymes in wheat. *Planta* 223:568-582
- 408 Böttcher C, Keyzers RA, Boss PK, Davies C (2010) Sequestration of auxin by the indole-3-acetic acid-amido
 409 synthetase GH3-1 in grape berry (*Vitis vinifera* L.) and the proposed role of auxin conjugation during ripening. *J*
 410 *Exp Bot* 61:3615-3625
- 411 Brugiere N, Jiao S, Hantke S, Zinselmeier C, Roessler JA, Niu X, Jones RJ, Habben JE (2003) Cytokinin oxidase
 412 gene expression in maize is localized to the vasculature, and is induced by cytokinins, abscisic acid, and abiotic
 413 stress. *Plant Physiol* 132:1228-1240
- 414 Chiwocha, SDS, Abrams, SR, Ambrose, SJ, Cutler, AJ, Loewen, M, Ross, ARS, Kermode, AR (2003) A method for
 415 profiling classes of plant hormones and their metabolites using liquid chromatography-electrospray ionization
 416 tandem mass spectrometry: analysis of hormone regulation of thermodormancy of lettuce (*Lactuca sativa* L.)
 417 seeds. *Plant J* 35:405-417
- 418
 419 Chiwocha, SDS, Cutler, AJ, Abrams, SR, Ambrose, SJ, Yang, J, Ross, ARS, Kermode, AR (2005) The *etr1-2*
 420 mutation in *Arabidopsis thaliana* affects the abscisic acid, auxin, cytokinin and gibberellin metabolic pathways
 421 during maintenance of seed dormancy, moist-chilling and germination. *Plant J* 42:35-48
- 422 Croser JS, Lulsdorf MM, Davies PA, Clarke HJ, Bayliss KL, Mallikarjuna N, Siddique KHM (2006) Toward
 423 doubled haploid production in the *Fabaceae*: progress, constraints, and opportunities. *Crit Rev Plant Sci* 25:139-
 424 157
- 425 DeMason DA and Polowick PL (2009) Patterns of *DR5::GUS* expression in organs of pea (*Pisum sativum*).
 426 *International Journal of Plant Sciences* 170: 1 – 11
- 427 Dorcey E, Urbez C, Blazquez MA, Carbonell J, Perez-Amador MA (2009) Fertilization-dependent auxin response
 428 in ovules triggers fruit development through the modulation of gibberellin metabolism in *Arabidopsis*. *Plant J*
 429 58:318-332
- 430 Dunwell JM, Thurling N (1985) Role of sucrose in microspore embryo production in *Brassica napus* ssp. *oleifera*. *J*
 431 *Exp Bot* 36:1478-1491
- 432 Emery RJN, Lepoint L, Barton JE, Turner NC, Atkins CA (1998) *cis*-Isomers of cytokinins predominate in chickpea
 433 seeds throughout their development. *Plant Physiol* 117:1515-1523
- 434 Engvild KC, Egsgaard H, Larsen E (1981) Determination of 4-chloroindoleacetic acid methyl ester in *Viciae* species
 435 by gas chromatography-mass spectrometry. *Physiol Plant* 53:79-81
- 436 Ferrie AMR, Palmer CE, Keller WA (1995) Haploid embryogenesis. In: Thorpe TA, editor. *In vitro* embryogenesis
 437 in plants. Dordrecht: Kluwer Academic Publishers, pp 309-344

- 438 Ferrie AMR, Taylor DC, MacKenzie SL, Keller WA (1999) Microspore embryogenesis of high *sn-2* erucic acid
439 *Brassica oleracea* germplasm. *Plant Cell Tissue Organ Cult* 57:79–84
- 440 Fizzell LA (1988) Biological effects of acoustic cavitation. In: Suslick KS, editor. *Ultrasound. Its chemical, physical*
441 *and biological effects*. Weinheim VCH Publishers, pp 287-304
- 442 Fleet CM, Sun T (2005) A delicate balance: the role of gibberellin in plant morphogenesis. *Curr Opin Plant Biol*
443 8:77-85
- 444 Gao Y, Li T, Liu Y, Ren C, Zhao Y, Wang M (2010) Isolation and characterization of gene encoding G protein α
445 subunit protein responsive to plant hormones and abiotic stresses in *Brassica napus*. *Mol Biol Rep* 37:3957-
446 3965
- 447 Ghanashyam C, Jain M (2009) Role of auxin-responsive genes in biotic stress responses. *Plant Signaling &*
448 *Behaviour* 4:846-848
- 449 Grewal RK, Lulsdorf M, Croser J, Ochatt S, Vandenberg A, Warkentin TD (2009) Doubled-haploid production in
450 chickpea (*Cicer arietinum* L.): role of stress treatments. *Plant Cell Rep* 28:1289-1299
- 451 Hirano K, Aya K, Hobo T, Sakakibara H, Kojima M, Shim RA, Hasegawa Y, Ueguchi-Tanaka M, Matsuoka M
452 (2008) Comprehensive transcriptome analysis of phytohormone biosynthesis and signaling genes in
453 microspore/pollen and tapetum of rice. *Plant Cell Physiol* 49:1429-1450
- 454 Hoekstra S, van Zijderveld MH, Heidekamp F, van der Mark F (1993) Microspore culture of *Hordeum vulgare* L.:
455 the influence of density and osmolality. *Plant Cell Rep* 2:661-665
- 456 Imamura J, Harada H (1980) Effects of abscisic acid and water stress on the embryo and plantlet production in
457 anther culture of *Nicotiana tabacum* cv. Samsun. *Zeitschrift Pflanzenphysiol* 100:285-289
- 458 Jain M; Khurana JP (2009) Transcript profiling reveals diverse roles of auxin-responsive genes during reproductive
459 development and abiotic stress in rice. *FEBS J* 276:3148-3162
- 460 Jardinaud MF, Souvre A, Alibert G (1993) Transient GUS gene expression in *Brassica napus* electroporated
461 microspores. *Plant Sci* 93:177-184
- 462 Joersbo M, Brunstedt J (1992) Sonication: a new method for gene transfer to plants. *Physiol Plant* 85: 230–234
- 463 Kaltchuk-Santos E, Mariath J E, Mundstock E, Hu C and Bodanese-Zanettini M H (1997) Cytological analysis of
464 early microspore divisions and embryo formation in cultured soybean anthers. *Plant Cell, Tissue and Organ*
465 *Culture*, 49, 107-115.
- 466 Kikuchi A, Sanuki N, Higashi K, Koshiha T, Kamada H (2006) Absciscic acid and stress treatment are essential for
467 the acquisition of embryogenic competence by carrot somatic cells. *Planta* 223:637-645
- 468 Laurain D, Tremouillaux-Guiller J, Chenieux JC (1993) Embryogenesis from micropores of *Ginkgo biloba* L., a
469 medicinal woody species. *Plant Cell Rep* 12:501-505
- 470 Lionneton E, Beuret W, Delaitre C, Ochatt S, Rancillac M (2001) Improved microspore culture and doubled haploid
471 plant regeneration in the brown condiment mustard (*Brassica juncea*). *Plant Cell Rep* 20:126-130
- 472 Maraschin SF, Caspers M, Potokina E, Wulfert F, Graner A, Spaink HP, Wang M (2006) cDNA array analysis of
473 stress-induced gene expression in barley androgenesis. *Physiol Plant* 127:535-550
- 474 Maraschin SF, de Priester W, Spaink HP, Wang M (2005) Androgenic switch: an example of plant embryogenesis
475 from the male gametophyte perspective. *J Exp Bot* 56:1711-1726
- 476 Normanly J, Slovin JP, Cohen JD (1995) Rethinking auxin biosynthesis and metabolism. *Plant Physiol.* 107:323-329
- 477 Ochatt S, Chand P, Rech EL, Davey MR, Power JB (1988) Electroporation-mediated improvement of plant
478 regeneration from Colt cherry (*Prunus avium* x *pseudocerasus*) protoplast. *Plant Sci* 54:165-169
- 479 Ochatt S, Pech C, Grewal R, Conreux C, Lulsdorf M, Jacas L (2009) Abiotic stress enhances androgenesis from
480 isolated microspores of some legume species (*Fabaceae*). *J Plant Physiol* 166:1314-1328

- 481 Oetiker JH; Aeschbacher G (1997) Temperature-sensitive plant cells with shunted indole-3-acetic acid conjugation.
482 Plant Physiol 114:1385-1395
- 483 Ostrowski M and Jakubowska A (2011) Purification and biochemical characterization of indole-3-acetyl-aspartic
484 acid synthetase from immature seeds of pea (*Pisum sativum*). J Plant Growth Regul 30:30-40
- 485 Park S, Ozga JA, Cohen JD, Reinecke DM (2010) Evidence of 4-Cl-IAA and IAA bound to proteins in pea fruit. J
486 Plant Growth Regul 29:184-193
- 487 Park J-E, Park J-Y, Kim Y-S, Staswick PE, Jeon J, Yun J, Kim S-Y, Kim J, Lee Y-H, Park C-M (2007) GH3-
488 mediated auxin homeostasis links growth regulation with stress adaptation response in *Arabidopsis*. J Biol Chem
489 282 (13):10036-10046
- 490 Quesnelle E; Emery RJN (2007) Cis-cytokinins that predominate in *Pisum sativum* during early embryogenesis will
491 accelerate embryo growth *in vitro*. Can J Bot 85:91-103
- 492 Rashid A; Street HE (1974) Segmentations in microspores of *Nicotiana tabacum* which lead to embryoid formation
493 in anther cultures. Protoplasma 80:323-324
- 494 Rech EL, Ochatt S, Chand P, Power JB, Davey MR (1987) Electro-enhancement of division of plant protoplast-
495 derived cells. Protoplasma 141:169-176
- 496 Reinecke DM (1999) 4-Chloroindole-3-acetic acid and plant growth. Plant Growth Regul 27:3-13
- 497 Ribalta, F, Croser, J, Ochatt, S (2010) Doubled-haploid production in field pea via anther culture. Poster No. 54.
498 International Food Legume Conference, Antalya, Turkey
- 499 Ross ARS, Ambrose SJ, Cutler AJ, Feurtado JA, Kermode AR, Nelson K, Zhou R and Abrams SR (2004)
500 Determination of endogenous and supplied deuterated abscisic acid in plant tissues by high performance liquid
501 chromatography-electrospray ionization tandem mass spectrometry with multiple reaction monitoring. Analytical
502 Biochemistry 329: 324-333
- 503 Sáenz L, Azpeitia A, Oropeza C, Jones LH, Fuchsova K, Spichal L and Strnad M (2010) Endogenous cytokinins in
504 *Cocos nucifera* L. *in vitro* cultures obtained from plumular explants. Plant Cell Rep 29: 1227-1234
- 505 Sakakibara, H 2006. Cytokinins: activity, biosynthesis, and translocation. Annual Rev Plant Biol 57:431-449
- 506 Sangwan-Norreel B S (1977) Androgenic stimulating factors in anther and isolated pollen grain culture of *Datura*
507 *innoxia* Mill. J Exp Bot 28:843-852
- 508 Sasaki K, Shimomura K, Kamada H, Harada H (1994) IAA metabolism in embryogenic and non-embryogenic
509 carrot cells. Plant Cell Physiol 35 (8): 1159-1164
- 510 Segui-Simarro JM, Nuez F (2008) How microspores transform into haploid embryos: changes associated with
511 embryogenesis induction and microspore-derived embryogenesis. Physiol Plant 134:1-12
- 512 Shariatpanahi ME, Bal U, Heberle-Bors E, Touraev A (2006) Stresses applied for the re-programming of plant
513 microspores towards *in vitro* embryogenesis. Physiologia Plant 127:519-534
- 514 Simon S, Petrášek J (2011) Why plants need more than one type of auxin. Plant Sci 180:454-460
- 515 Smykal P (2000) Pollen embryogenesis - the stress mediated switch from gametophytic to sporophytic development.
516 Current status and future prospects. Biol Plant 4:481-489
- 517 Swain SM, Singh DP (2005) Tall tales from sly dwarves: novel functions of gibberellins in plant development.
518 Trends Plant Sci 10:123-129
- 519 Tanaka M (1973) The effect of centrifugal treatment on the emergence of plantlets from cultured anthers of tobacco.
520 Japan J Breed 23:171-174
- 521 Tret'yakova IN, Ivanitskaya AC, Ivanova AN, Barsukova AV (2009) Phytohormone content in microstrobiles and
522 androgenetic callus of Siberian larch. Russ J Plant Physiol 56:647-653
- 523 Tromas A, Perrot-Rechenmann C (2010) Recent progress in auxin biology. Comptes Rendus Biol 333: 297-306
- 524 van Bergen S, Kottenhagen MJ, van der Meulen RM, Wang M (1999) The role of abscisic acid in induction of
525 androgenesis: a comparative study between *Hordeum vulgare* L. cvs. Igri and Digger. J Plant Growth Regul
526 18:135-143

- 527 Wang M, Hoekstra S, van Bergen S, Lamers GE, Oppedijk BJ, van der Heijden MW, de Priester W, Schilperoort
528 RA (1999) Apoptosis in developing anthers and the role of ABA in this process during androgenesis in *Hordeum*
529 *vulgare* L. Plant Mol Biol 39:489-501
- 530 Wedzony M, Forster BP, Żur I, Golemić E, Szechyńska-Hebda M, Dubas E, Gotebiowska G (2009) Progress in
531 doubled haploid technology in higher plants. In: Advances in haploid production in higher plants. Touraev A,
532 Forster BP, Jain SM (eds), Springer Science, pp 1-33
- 533 Werner T, Schmülling T (2009) Cytokinin action in plant development. Current Opinion Plant Biol 12: 527-538
- 534 Zaharia LI, Galka MM, Ambrose SJ, Abrams SR (2005) Preparation of deuterated abscisic acid metabolites for use
535 in mass spectrometry and feeding studies. J Labelled Compd Radiopharmaceut 48:435-445
- 536 Zaki MAM, Dickinson HG (1990) Structural changes during the first divisions of embryos resulting from anther and
537 free microspore culture in *Brassica napus*. Protoplasma 56:149-162
- 538 Zavattieri MA, Frederico AM, Lima M, Sabino R and Arnholdt-Schmitt B (2010) Induction of somatic
539 embryogenesis as an example of stress-related plant reactions. Electronic Journal of Biotechnology [online].
540 January 15, 2010, vol. 13, no. 1 [cited date]. ISSN 0717-3458 Available from Internet:
541 <http://www.ejbiotechnology.cl/content/vol13/issue1/full/4/index.html>
- 542 Zhao Y (2010) Auxin biosynthesis and its role in plant development. Annual Rev Plant Biol 61:49-64
- 543 Żur I, Dubas E, Golemić E, Szechyńska-Hebda M, Janowiak F, Wędzony M (2008) Stress-induced changes
544 important for effective androgenic induction of isolated microspore culture of triticale (*x Triticosecale* Wittm.)
545 Plant Cell, Tissue Organ Cult 94:319-328
546

Table 1. Content of ABA and its metabolites in nM g⁻¹ DW in anthers after cold, centrifugation (C), electroporation (E), sonication (S), or osmotic shock (O). Data presented are means $\pm$ SE and n indicates number of replicates per treatment. ND not detected

Species & Cultivar	Treatments	n	ABA	t-ABA	DPA	PA	neo-PA	ABA-GE	7'OH-ABA
<i>Pisum</i> CDC April	Control	2	2.14 $\pm$ 0.03	1.55 $\pm$ 0.07	131.03 $\pm$ 9.78	34.57 $\pm$ 6.73	0.62 $\pm$ 0.00	0.14 $\pm$ 0.01	0.12 $\pm$ 0.02
	Cold	1	0.51	1.40	41.08	173.19	0.14	ND	ND
	Cold +C	2	0.46 $\pm$ 0.02	2.65 $\pm$ 0.12	13.04 $\pm$ 2.06	1.65 $\pm$ 0.96	0.10 $\pm$ 0.01	ND	ND
	Cold +E	2	0.37 $\pm$ 0.00	0.80 $\pm$ 0.06	15.19 $\pm$ 0.98	1.04 $\pm$ 0.02	0.10 $\pm$ 0.00	0.09 $\pm$ 0.01	0.03 $\pm$ 0.03
	Cold +S	2	0.23 $\pm$ 0.00	0.54 $\pm$ 0.04	15.52 $\pm$ 0.30	2.37 $\pm$ 1.10	0.06 $\pm$ 0.01	0.20 $\pm$ 0.05	ND
	Cold +C+E	2	0.21 $\pm$ 0.05	1.04 $\pm$ 0.13	12.46 $\pm$ 1.83	19.39 $\pm$ 18.63	0.02 $\pm$ 0.02	0.10 $\pm$ 0.01	ND
	Cold +C+E+S	2	0.24 $\pm$ 0.04	0.90 $\pm$ 0.16	11.51 $\pm$ 0.82	9.72 $\pm$ 8.98	0.09 $\pm$ 0.04	0.05 $\pm$ 0.05	ND
	Cold +O	3	0.24 $\pm$ 0.02	0.17 $\pm$ 0.02	0.36 $\pm$ 0.05	0.17 $\pm$ 0.02		0.37 $\pm$ 0.00	ND
	Cold+C+E+S+O	1	ND	0.03	0.03	ND	ND	0.02	ND
<i>Pisum</i> Frisson	Control	2	2.61 $\pm$ 0.16	0.72 $\pm$ 0.00	35.13 $\pm$ 1.96	189.10 $\pm$ 71.57	0.41 $\pm$ 0.00	0.07 $\pm$ 0.02	0.02 $\pm$ 0.02
	Cold	1	0.37	1.61	38.11	33.81	0.16	0.05	ND
	Cold +E	2	0.32 $\pm$ 0.00	0.97 $\pm$ 0.02	22.39 $\pm$ 0.88	18.71 $\pm$ 0.95	0.05 $\pm$ 0.01	0.08 $\pm$ 0.01	ND
	Cold +C	3	0.30 $\pm$ 0.01	1.16 $\pm$ 0.03	51.18 $\pm$ 3.15	14.62 $\pm$ 3.34	0.12 $\pm$ 0.00	0.06 $\pm$ 0.03	0.02 $\pm$ 0.02
	Cold +S	2	0.29 $\pm$ 0.02	0.94 $\pm$ 0.04	28.28 $\pm$ 1.51	16.13 $\pm$ 2.69	0.07 $\pm$ 0.00	0.09 $\pm$ 0.00	ND
	Cold +C+E	3	0.54 $\pm$ 0.01	0.90 $\pm$ 0.04	24.93 $\pm$ 1.41	5.53 $\pm$ 0.80	0.14 $\pm$ 0.02	0.15 $\pm$ 0.01	0.02 $\pm$ 0.02
	Cold +C+E+S	3	0.27 $\pm$ 0.00	1.21 $\pm$ 0.05	43.63 $\pm$ 2.63	9.05 $\pm$ 1.15	0.10 $\pm$ 0.00	0.02 $\pm$ 0.02	ND
	Cold +O	2	0.07 $\pm$ 0.01	0.06 $\pm$ 0.03	0.05 $\pm$ 0.05	0.12 $\pm$ 0.00	ND	0.16 $\pm$ 0.03	ND
	Cold+C+E+S+O	2	0.09 $\pm$ 0.03	0.01 $\pm$ 0.01	0.11 $\pm$ 0.03	0.14 $\pm$ 0.02	ND	0.19 $\pm$ 0.02	0.01 $\pm$ 0.01
<i>Cicer</i> CDC Xena	Control	4	10.38 $\pm$ 0.98	1.19 $\pm$ 0.13	4.32 $\pm$ 0.77	26.12 $\pm$ 6.12	0.40 $\pm$ 0.11	0.53 $\pm$ 0.26	0.33 $\pm$ 0.17
	Cold	3	5.11 $\pm$ 0.66	1.10 $\pm$ 0.12	6.28 $\pm$ 1.15	21.27 $\pm$ 5.61	0.53 $\pm$ 0.05	0.62 $\pm$ 0.18	0.43 $\pm$ 0.04
	Cold +C	2	2.02 $\pm$ 0.95	1.10 $\pm$ 0.04	12.22 $\pm$ 0.51	112.12 $\pm$ 41.43	0.38 $\pm$ 0.05	0.18 $\pm$ 0.02	0.17 $\pm$ 0.05
	Cold +E	2	2.43 $\pm$ 0.77	1.13 $\pm$ 0.35	13.80 $\pm$ 4.00	105.74 $\pm$ 44.31	0.48 $\pm$ 0.01	0.18 $\pm$ 0.05	0.24 $\pm$ 0.03
	Cold +E+O	3	0.68 $\pm$ 0.21	0.12 $\pm$ 0.01	1.19 $\pm$ 0.17	1.34 $\pm$ 0.27	0.02 $\pm$ 0.01	0.58 $\pm$ 0.19	0.06 $\pm$ 0.03
	Cold +C+O	3	0.48 $\pm$ 0.12	0.06 $\pm$ 0.03	1.02 $\pm$ 0.09	1.41 $\pm$ 0.11	0.03 $\pm$ 0.02	0.54 $\pm$ 0.14	0.06 $\pm$ 0.03
	Cold +O	3	1.45 $\pm$ 0.30	0.19 $\pm$ 0.05	1.48 $\pm$ 0.54	1.80 $\pm$ 0.43	0.05 $\pm$ 0.01	1.38 $\pm$ 0.15	0.18 $\pm$ 0.03

	Cold +O	2	1.76±0.18	6.09±0.49	0.13±0.00	ND
	Cold +C+E+S+O	2	1.45±0.32	5.45±0.93	0.04±0.04	ND
Cicer	Control	4	0.61±0.07	0.07±0.02	ND	ND
CDC	Cold	3	0.42±0.04	0.04±0.04	ND	ND
Xena	Cold +C -IAA	1	0.43	0.04	ND	ND
	Cold +C +IAA	1	5.57	1.45	0.04	ND
	Cold +E -IAA	1	0.14	0.07	ND	ND
	Cold +E+IAA	1	3.37	0.64	ND	ND
	Cold+O	3	3.30±0.62	1,219.12±155.30	39.44± 06.30	0.14±0.07
	Cold +C+O	3	5.10±2.88	1,284.24±226.47	60.77±12.27	0.35±0.17
	Cold +E+O	3	2.04±0.18	971.13±310.39	33.17±06.47	0.15±0.03
	Cold +C+E+O		2.63±1.18	1,022.88±322.39	35.94±14.25	0.08±0.05
Lens	Control	1	1.02	1.29	0.03	ND
CDC	3 d Cold	1	1.26	0.95	ND	ND
Maxim	7d Cold	2	3.07±0.39	1.25±0.15	0.01± 0.01	ND
	O+ 3d Cold	2	1.00±0.12	0.66±0.08	0.01± 0.01	ND
	E+O+3d Cold	2	0.82±0.18	0.62±0.06	ND	ND
	C+O+3d Cold	2	0.78±0.03	0.66±0.05	ND	ND

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Table 3. Content of cytokinin and its metabolites in nM g⁻¹ DW in anthers after cold, centrifugation (C), electroporation (E), sonication (S), or osmotic shock (O). Data presented are means ±SE and n indicates number of replicates per treatment. ND not detected

Species & Cultivar	Treatments	n	c-ZR	2IP	iPA	c-Z	t-Z	t-ZR	dhZ	dh-ZR
Pisum	Control ²	2	0.58±0.01	0.87±0.03	0.20±0.01	0.05±0.00	0.01±0.01	0.05±0.00	ND	ND
CDC	Cold	1	0.9	0.67	0.1	0.01	ND	ND	ND	ND
April	Cold +C	2	1.84±0.01	0.53±0.03	0.10±0.00	ND	ND	ND	ND	ND
	Cold +E	2	0.62±0.02	0.50±0.02	0.08±0.00	0.01±0.01	ND	ND	ND	ND
	Cold +S	2	0.36±0.01	0.63±0.01	0.05±0.00	0.01±0.01	ND	ND	ND	ND

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	Cold +C+E ²	2	0.86±0.09	0.67±0.07	0.09±0.00	0.01±0.01	ND	ND	ND	ND
	Cold +C+E+S	2	0.81±0.06	0.65±0.07	0.10±0.01	ND	ND	ND	ND	ND
	Cold +O	3	0.14±0.00	0.04±0.00	0.04±0.01	ND	0.01±0.01	ND	0.03±0.01	ND
	Cold+C+E+S+O	1	0.02	0.08	0.02	0.02	ND	ND	ND	ND
Pisum	Control	2	0.16±0.01	0.82±0.03	0.26±0.06	0.03±0.00	ND	ND	ND	ND
Frisson	Cold	1	1.15	0.87	0.2	ND	ND	ND	ND	ND
	Cold +C	3	1.04±0.05	0.80±0.03	0.14±0.00	ND	ND	ND	ND	ND
	Cold +E	2	1.09±0.05	0.88±0.01	0.14±0.00	0.01±0.01	ND	ND	ND	ND
	Cold +S	2	1.17±0.08	0.88±0.03	0.16±0.01	ND	ND	ND	ND	ND
	Cold +C+E	3	0.67±0.00	0.68±0.03	0.09±0.01	ND	ND	ND	ND	ND
	Cold +C+E+S	3	1.06±0.03	0.82±0.02	0.16±0.00	ND	ND	ND	ND	ND
	Cold +O	2	0.06±0.04	0.01±0.01	0.01±0.01	ND	ND	ND	ND	ND
	Cold+C+E+S+O	2	0.01±0.01	0.01±0.01	0.01±0.01	ND	ND	ND	ND	ND
Cicer	Control	4	0.80±0.22	0.04±0.05	0.39±0.12	ND	0.25±0.04	1.18±0.26	0.16±0.04	0.27±0.08
CDC	Cold	3	1.02±0.29	ND	0.23±0.02	ND	0.12±0.01	1.08±0.12	0.10±0.02	0.47±0.05
Xena	Cold +C	2	2.86±1.06	0.05±0.01	0.94±0.33	ND	0.15±0.00	0.60±0.01	0.15±0.02	0.39±0.03
	Cold +E	2	3.14±0.54	0.06±0.03	0.88±0.17	ND	0.12±0.02	0.46±0.05	0.12±0.02	0.28±0.09
	Cold +C+O	3	0.12±0.05	ND	ND	ND	ND	ND	ND	0.02±0.02
	Cold +E+O	3	0.12±0.06	0.02±0.02	ND	ND	ND	ND	ND	0.01±0.01
	Cold +O	3	0.15±0.07	ND	ND	ND	ND	ND	ND	0.00±0.00
	Cold+C+E+O	3	0.13±0.05	ND	ND	ND	ND	ND	ND	0.01±0.01
Lens	Control	1	1.04	0.35	0.1	ND	ND	ND	ND	ND
CDC	3 d Cold	1	1.16	0.33	0.18	ND	ND	ND	ND	ND
Maxim	7d Cold	1	0.25±0.01	0.24±0.01	0.01±0.01	ND	ND	ND	ND	ND
	O+ 3d Cold	2	0.41±0.01	0.16±0.00	0.04±0.00	ND	ND	ND	ND	ND
	E+O+3d Cold	2	0.32±0.02	0.12±0.00	0.03±0.00	ND	ND	ND	ND	ND
	C+O+3d Cold	2	0.15±0.02	0.14±0.01	0.02±0.00	ND	ND	ND	ND	ND

Table 4. Content of gibberellin and its metabolites in nM g^{-1} DW in anthers after cold, centrifugation (C), electroporation (E), sonication (S), or osmotic shock (O). Data presented are means $\pm$ SE and n indicates number of replicates per treatment. ND not detected

Species & Cultivar	Treatments	n	GA ₁₉	GA ₂₀	GA ₈	GA ₉	GA ₂₉	GA ₄₄	GA ₅₃
<i>Pisum</i>	Control	2	0.81 $\pm$ 0.01	3.41 $\pm$ 0.06	ND	0.66 $\pm$ 0.05	ND	0.12 $\pm$ 0.03	0.08 $\pm$ 0.01
CDC	Cold	1	0.99	0.25	ND	ND	ND	ND	ND
April	Cold +C	2	1.63 $\pm$ 0.05	ND	ND	ND	ND	ND	ND
	Cold +E	2	1.04 $\pm$ 0.02	0.11 $\pm$ 0.01	ND	ND	ND	ND	ND
	Cold +S	2	1.98 $\pm$ 0.06	ND	ND	ND	ND	ND	0.11 $\pm$ 0.01
	Cold +C+E	2	1.14 $\pm$ 0.14	ND	ND	ND	ND	ND	0.02 $\pm$ 0.02
	Cold +C+E+S	2	1.34 $\pm$ 0.25	ND	ND	ND	ND	ND	0.08 $\pm$ 0.05
	Cold +O	3	0.37 $\pm$ 0.02	ND	ND	ND	ND	ND	ND
	Cold +C+E+S+O	1	0.05	ND	ND	ND	ND	ND	ND
<i>Pisum</i>	Control	2	0.40 $\pm$ 0.01	2.31 $\pm$ 0.08	ND	3.77 $\pm$ 0.20	2.89 $\pm$ 2.89	0.10 $\pm$ 0.00	0.01 $\pm$ 0.01
Frisson	Cold	1	1.93	0.11	ND	ND	ND	ND	0.17
	Cold +C	3	1.00 $\pm$ 0.03	ND	ND	ND	ND	ND	0.06 $\pm$ 0.01
	Cold +E	2	1.82 $\pm$ 0.08	0.04 $\pm$ 0.04	ND	ND	ND	ND	0.08 $\pm$ 0.01
	Cold +S	2	1.38 $\pm$ 0.13	ND	ND	ND	ND	ND	0.06 $\pm$ 0.00
	Cold +C+E	3	1.12 $\pm$ 0.01	ND	ND	ND	ND	ND	0.01 $\pm$ 0.01
	Cold +C+E+S	3	1.07 $\pm$ 0.06	0.03 $\pm$ 0.03	ND	ND	ND	ND	0.02 $\pm$ 0.02
	Cold +O	2	0.06 $\pm$ 0.00	ND	ND	ND	ND	ND	ND
	Cold +C+E+S+O	2	0.04 $\pm$ 0.04	ND	ND	ND	ND	ND	ND
<i>Cicer</i>	Control	4	0.28 $\pm$ 0.06	2.62 $\pm$ 0.51	1.75 $\pm$ 0.32	0.09 $\pm$ 0.07	0.26 $\pm$ 0.20	ND	0.03 $\pm$ 0.02
CDC	Cold	3	0.75 $\pm$ 0.18	1.17 $\pm$ 0.24	1.13 $\pm$ 0.09	ND	0.71 $\pm$ 0.36	ND	0.18 $\pm$ 0.06
Xena	Cold +C	2	0.72 $\pm$ 0.16	1.17 $\pm$ 0.50	0.87 $\pm$ 0.12	ND	ND	ND	0.20 $\pm$ 0.01
	Cold +E	2	0.95 $\pm$ 0.07	0.93 $\pm$ 0.61	0.73 $\pm$ 0.30	ND	ND	ND	0.14 $\pm$ 0.04
	Cold +C+O	3	0.07 $\pm$ 0.02	ND	ND	ND	ND	ND	ND
	Cold +E+O	3	0.08 $\pm$ 0.04	ND	ND	ND	ND	ND	ND
	Cold +O	3	0.02 $\pm$ 0.02	ND	ND	ND	ND	ND	ND
	Cold +C+E+O	3	0.07 $\pm$ 0.04	ND	ND	ND	ND	ND	ND

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<i>Lens</i>	Control	1	0.48	0.5	0.94	ND	1.48	1.39	0.15
CDC	3 d Cold	1	0.47	ND	0.32	ND	ND	0.26	0.27
Maxim	7d Cold	2	0.26±0.03	ND	ND	ND	ND	ND	0.04±0.04
	O+3d Cold	2	0.40±0.03	ND	0.25±0.00	ND	ND	0.30±0.01	0.11±0.02
	E+O + 3d Cold	2	0.18±0.06	ND	ND	ND	ND	0.05±0.05	0.02±0.02
	C+O + 3d Cold	2	0.10±0.06	ND	ND	ND	ND	0.04±0.04	ND