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ORIGINAL PAPER

Precocious floral initiation and identification of exact timing of embryo physiological maturity facilitate germination of immature seeds to truncate the lifecycle of pea

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Abstract We propose herein a novel single seed descent protocol that has application across a broad phenotypic range of pea genotypes. Manipulation of key in vivo growing conditions, including light, photoperiod and temperature, combined with precocious in vitro germination of the embryo at full physiological maturity substantially shortened the pea lifecycle. We define full embryo physiological maturity as the earliest point in seed development when precocious in vitro germination and robust seedling growth can be reliably achieved without supply of exogenous hormones. Under our optimised conditions for accelerated plant growth, embryo physiological maturity was attained at *c*. 18 days after pollination, when seed moisture content was below 60 % and sucrose level under 100 mg g^{-1} DW. No delay penalty in terms of time to flowering and plant development was caused by the culture of immature seeds 18 days after pollination compared to the used of mature ones. Determining the role embryo maturity plays in the fitness of the germinated plant has facilitated the truncation of the lifecycle across pea genotypes. The accelerated single seed descent system proposed within this research will benefit complex genetic studies via the rapid development of recombinant inbred lines (RIL) and multi-parental advanced generation intercrosses (MAGIC) populations.

Keywords Early floral onset · Embryo physiological maturity · *Pisum sativum* L. · Precocious seed germination · Seed moisture content · Seed sucrose content

Introduction

The current extended generation cycle time is a serious impediment to progress in the genetic enhancement of pea (*Pisum sativum* L.). To improve pea cultivars, landraces or primitive forms are hybridised, elite individuals selected and genes fixed using pedigree, bulk, backcross or single seed descent (SSD) methods (Redden et al. 2005). Depending on infrastructure resources, pea geneticists can achieve between one field-based generation and three glasshouse-based generations per year. Pea and the other *Fabaceae* species lag behind cereals and oilseeds in the availability of time-saving technology such as doubled haploidy, which permits the development of homozygous individuals from gametes in a single generation (Maluszynski et al. 2003; Croser et al. 2006; Germanà 2011).

Within this context, we set out to determine whether the generation cycle could be radically shortened in pea by understanding and then manipulating: (1) the physiology of flowering response, particularly in late and very late flowering genotypes, and (2) the precise time/developmental stage at which the embryo achieves germination competence with low external input. The literature provides compelling evidence to suggest combining in vivo growth conditions designed to trigger early flowering with technology to enable precocious germination of immature seed will enable

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development of a robust and genotype-independent protocol for rapid generation cycling (Ochatt et al. 2002; Ochatt and Sangwan 2010).

Conventional pea genetic improvement activities use SSD to rapidly fix genes in breeding lines. This technique involves the advancement of a single randomly-selected seed per plant from early segregating generations (Goulden 1939; Brim 1966). As only one seed is used to produce the next generation, plants can be grown under conditions that accelerate flowering and seed set, but do not encourage high yield, enabling up to three generations per year in pea (Ochatt et al. 2002). Two in vitro based strategies have been proposed to accelerate SSD turnover in early flowering pea genotypes: (1) alternating between in vivo growth and in vitro germination of immature seed to produce 5–5.5 generations per year (Ochatt et al. 2002), and (2) completing the full life cycle in vitro to produce up to 6.8 generations per year (Ochatt et al. 2002; Franklin et al. 2000). To develop a genotype-independent technique with wider applicability to genetic enhancement, Ribalta et al. (2014) reported a modified in vitro SSD system which encompassed mid to late flowering types. However, some late and very late flowering genotypes remained unresponsive to the triggering of early flowering in vitro, limiting its general use within breeding programs. This genotype-specificity led us to conclude that a two-phase approach of in vivo plant growth followed by a short in vitro period for precocious germination, initially proposed by Ochatt et al. (2002), is the most promising for the successful development of a genotype-independent protocol for rapid generation turnover. To modify the protocol to achieve genotype-independence, we explored mechanisms for precocious floral initiation in plants with late and very late flowering phenology under normal environments.

Floral transition in pea has been widely studied and is determined by environmental conditions of which light (quality and intensity), day length and temperature are most important (Murfet and Reid 1974; Moe and Heins 1990; Nelson et al. 2010). Light provides environmental information for the plant and consequently affects a wide range of photomorphogenic responses including flowering (Zhou and Singh 2002; Spalding and Folta 2005). In pea, a long-day plant, long photoperiods promote flowering by reducing the synthesis or transport of an inhibitor of flowering. This promotive effect is predominantly achieved through a response that requires long exposure to light and for which far-red (FR) light is most effective (Weller et al. 1997). In long-day plants, a red to far-red (R:FR) ratio close to the natural daylight level of 'around 1' is most effective for floral induction (Vince-Prue 1981). Temperature is the most important factor controlling the rate of plant development. Vernalisation notwithstanding, temperatures greater than a critical minimum base to an upper maximum will shorten developmental phases in plants, reducing the crop life cycle (Vadez et al. 2012; Iannucci et al. 2008). It is our intention to exploit this information to identify in vivo growth conditions that will reliably trigger precocious floral initiation across the full range of pea breeding germplasm in Australia.

To further accelerate the generation cycle, we need to identify the earliest point during pea seed development when maximum in vitro germination and optimal seedling growth can be reliably achieved with low external inputs. Legume seed development can be divided into three phases: (1) cell division (embryogenic axis development), (2) seed filling (physiological maturation) and (3) dehydration and acquisition of desiccation tolerance (Weber et al. 2005). The acquisition of germination competence is associated with an increase in seed dry matter during phase 2 when all embryo structures have been formed (Gallardo et al. 2003). Embryo physiological maturity is achieved at the end of the seed-filling phase when seed reaches its maximum dry weight (Ellis et al. 1987). Factors implicated in the modulation of pea embryo physiological maturity include seed moisture content and sucrose concentration (Le Deunff and Rachidian 1988; Ellis et al. 1987; Weber et al. 2005). In pea, embryo physiological maturity is reached when the seed moisture content level drops below 55 % during phase 2, and optimal seed germination and subsequent seedling growth are achieved (Le Deunff and Rachidian 1988; Ellis et al. 1987). Clear data for sucrose content in pea is unavailable, but for the grain legume *Vicia narbonesis*, Weber et al. (1998) demonstrated peak sucrose concentration as the point at which cells start to actively accumulate starch, which is a reliable indicator of the onset of embryo maturation. Using these results as a starting point, we intend to establish indicators of embryo maturation through in vitro germination of pea embryos at different developmental stages. The development of simple, reliable indicators of embryo physiological maturity in pea will assist in the identification of the most appropriate stage for rapid in vitro germination, and also in the transfer of this technology to other economically-important species.

Based on the literature we expect that optimising in vivo growth parameters, including light, photoperiod duration and temperature, will induce early floral onset. Combining this with the identification of the exact timing of physiological maturity of the embryo that facilitates robust in vitro germination and seedling growth should enable us to overcome previous constraints of genotype dependence.

Materials and methods

The research was undertaken in the controlled plant growth facilities at The University of Western Australia, Perth (lat: 31°58′49″S; long: 115°49′7″E). Three cultivars representing early (PBA Twilight), mid (PBA Pearl) and late (Kaspa) field

flowering phenology were used for the initial experiments on precocious floral initiation. These genotypes were also used to determine sucrose and moisture contents indicating embryo physiological maturity and in vitro germination competence (Table 1). To develop a genotype-independent in vivo/in vitro generation acceleration protocol, a broad range of early, mid, late and very late flowering pea genotypes representative of modern Australian breeding material was used (Table 1). This research included: (1) development of the in vitro germination protocol and (2) determination of the effect of precocious germination on phenology.

Study of key growth parameters for rapid in vivo floral onset

To determine the most appropriate conditions for early floral onset, plants of the cultivars PBA Twilight (early), PBA Pearl (mid) and Kaspa (late) were grown in a range of environments with different physical conditions (Table 2) and compared with field data (Merredin trial station, lat: 31°28′55″S; long: 118°16′44″E; Department of Agriculture and Food Western Australia). The R:FR ratio in Environment 4 was adjusted to closely mimic the natural light ratio. Light measurements were undertaken in all tested environments using a Sekonic C7000 SpectroMaster spectrometer (Sekonic Corp., Tokyo, Japan). Light intensity under natural conditions (Environments 1, 2 and 3) was measured during October and November at three different times (0800, 1200 and 1600 h) to check variation throughout the day. These values were averaged over three scans in the range of 380–780 nm. Ratio calculations followed the method of Runkle and Heins (2001) where the R:FR ratio was measured as a narrow band (655–665:725–735 nm). Seeds were sown in plastic 1 L pots filled with steam pasteurised potting mix (UWA Plant Bio Mix—Richgro Garden Products Australia Pty Ltd). Plants were watered daily and fertilised weekly with a water soluble N:P:K fertiliser (19:8.3:15.8) with micronutrients (Poly-feed, Greenhouse Grade, Haifa Chemicals Ltd.) at a rate of 2 g per pot. Flowering time (days from sowing to anthesis of the first flower) was recorded under different growth conditions. Anthesis (pollination) was recorded when petals extended beyond the sepals.

Table 2 The range of environments and growing conditions used for early flowering induction

	Environment 1	Environment 2	Environment 3	Environment 4
Temperature (day/night)	20/18 °C	24/20 °C	24/20 °C	24/20 °C
Light source	Natural light	Natural light	Natural light plus incandescent globes[a] for 8 h day^{-1}	Metal halide lamps[b] and incandescent globes[a]
R:FR ratio	1.2	1.2	1.2	1.3
Light intensity (μmol m^{-2} s^{-1})	0800 h: 500–700 1200 h: 900–1000 1600 h: 600–800			600 (constant)
Photoperiod	13–14 h	13–14 h	20 h	20 h

[a]Philips Eco Classic globes, 42 W 240 V

[b]400 W Uni-form Pulse Start, Venture Lighting Int

Identification of the earliest embryo developmental stage at which maximum in vitro germination and optimal seedling growth occur

To identify when reliable in vitro germination and optimal seedling growth occurred with low external inputs (i.e. no plant growth regulators required), the sucrose concentration and moisture content were measured in immature seeds produced in Environment 4 across a range of time intervals. Flowers of PBA Twilight (early), PBA Pearl (mid) and Kaspa (late) were individually tagged at anthesis. Samples of developing seeds were collected every 2 days from 10 to 30 DAP (days after pollination) and the integuments removed. Sucrose and moisture content analysis from immature seeds

Table 1 Pea genotypes used in this study and their main characteristics

Genotype	Flowering type	Leaf type	Plant size	Comment
PBA Twilight[a]	Early	Semi-leafless	Medium-tall	Australian cv.—Kaspa type
PBA Pearl[a]	Mid	Semi-leafless	Medium-tall	Australian cv.—grain yield type
PBA Hayman	Late	Conventional	Tall	Australian cv.—forage type
PBA Coogee	Late	Conventional	Tall	Australian cv.—forage/grain type
OZP1202	Late	Semi-leafless	Semi-dwarf	Australian line—Kaspa type
OZP1101	Late	Semi-leafless	Semi-dwarf	Australian line—Kaspa type
Kaspa[a]	Late	Semi-leafless	Semi-dwarf	Australian cv.—industry standard
00P016-1	Very late	Conventional	Tall	Ethiopian line—blackspot resistance

[a]Used as representative of early, mid and late flowering phenology in initial experiments

were undertaken in Environment 1 as a control. This analysis was carried out in a narrower range which comprises the period between acquisition of germination capacity (14 DAP) and full embryo maturation (22 DAP) (Gallardo et al. 2008; Weber et al. 2005), to characterise and compare immature seed development in a less optimal environment. All samples were stored at −80 °C until being freeze-dried at 20 µbar and 22 °C using a VirTis®, Bench Top™ K series freeze dryer (Gardiner, NY, USA).

Moisture content (%) was calculated as the difference in weight before and after sample freeze-drying and expressed as a percentage of fresh weight. For the sucrose quantification analysis, 0.1–1.0 g of freeze-dried material was weighed and placed into a 15 ml falcon tube with 10 ml of 80 % (v/v) methanol. The tissue was then homogenised and kept at 4 °C overnight to extract. Labelled internal standard ($^{13}C_{12}$ sucrose, Omicron Biochemicals, South Bend, IN, USA) was added to 20 µl of sample, taken up in 2 % acetic acid in water and then centrifuged for 3 min at 13,000 g. Samples were analysed by UPLC-MS as described previously (Mason et al. 2014).

In vivo/in vitro accelerated single seed descent (aSSD) protocol development

Plants of the eight phenotypically-diverse pea genotypes (Table 1) were grown under the most inductive conditions for flowering (Environment 4; Table 2) and flowers were individually tagged at anthesis (as previously described). Pods containing immature seeds were harvested at 14, 16 and 18 DAP and surface-sterilised in 70 % (v/v) ethanol for 1 min. Pods were opened under sterile conditions and immature seeds (3–4 seeds per pod), without integuments, were cultured in 80 mm Petri dishes (5 seeds per dish) containing 25 ml of modified MS medium (MS-2; Murashige and Skoog 1962) supplemented with 2 % sucrose (Sigma), 0.6 % agar (Type M, Sigma) and a pH of 5.6. The MS-2 medium was autoclaved at 121 °C for 20 min. A minimum of 50 seeds per genotype per treatment was cultured. Seeds were incubated at 25 °C in the dark for the first 24 h and then transferred to a 16 h photoperiod with a light intensity of 100 µmol m^{-2} s^{-1} from cool white fluorescent tubes (LIFEMAX, TL-D 3-W/840, Philips Lighting, Bangkok, Thailand) for another 72 h.

Seedlings were individually transplanted to 48-well plastic trays (Rite Gro—Garden City Plastics, Perth, Australia) filled with steam pasteurised potting mix and grown under the same conditions as the parent plants. Plants were hardened by placing them at high humidity for 1 week. 2 weeks after transplanting, ten seedlings per genotype from the 16 and 18 DAP developmental stages were transferred to 1 L pots and taken to the next generation. For all treatments, the percentages of germination after 4 days of in vitro culture and plants at the third-leaf stage after 14 days of sowing, and the time to flowering were recorded. Embryos were considered germinated when both radicle and shoot emergence was observed. To show the seed development in Environment 4, photos of immature seeds of the cultivar Kaspa were taken every 2 days from 14 to 22 DAP using an Axiocam camera and annotated using Axiovision Imaging System software (Carl Zeiss, Germany), and fresh weight recorded.

The experimental design was completely randomised and all treatments were repeated at least twice. Statistical analysis of the percentage germination after 4 days of culture and the percentage of plants that reached, at least, the third-leaf stage was performed using χ^2 variance test for homogeneity of the binomial distribution. Other statistical analyses were performed by ANOVA. The significant difference level for χ^2 and ANOVA was set at $P \leq 0.05$. Moisture content values (%) in Fig. 2 were arcsine transformed prior to statistical tests to improve normality. Means were compared using Tukey's test and standard error values calculated using IBM SPSS Statistics 22.0 software.

Results

Study of key growth parameters for rapid in vivo floral onset

For the early (PBA Twilight), mid (PBA Pearl) and late (Kaspa) flowering cultivars, time to flower initiation differed across genotypes and environments and was modulated by temperature, light and photoperiod. The R:FR ratio, known key factor in the induction of early flowering in pea (Cummings et al. 2007), was adjusted to closely mimic natural light in Environment 4 (Table 2). All four environmental regimes accelerated floral onset compared to field data (Fig. 1). For the three genotypes tested, floral initiation was most precocious when plants were grown under a photoperiod of 20 h, a temperature of 24/20 °C (day/night) and a constant light intensity of 600 µmol m^{-2} s^{-1} provided by a combination of metal halide lamps and incandescent lights (Environment 4). These conditions resulted in 2.4–2.5 times faster floral onset compared to field conditions. Growing plants under natural light conditions, ambient photoperiod of 13–14 h and a temperature of 20/18 °C (day/night; Environment 1) delayed floral onset for the three pea cultivars compared to the other three environments. As expected, floral onset was accelerated by increasing the growing temperature (Environment 2; between 6.5 and 10 days) and extending the photoperiod with the addition of incandescent light (Environment 3; between 14 and 20.7 days). Environment 4 conditions accelerated flowering by 18.8–26.7 days compared to Environment 1 and was thus adopted for

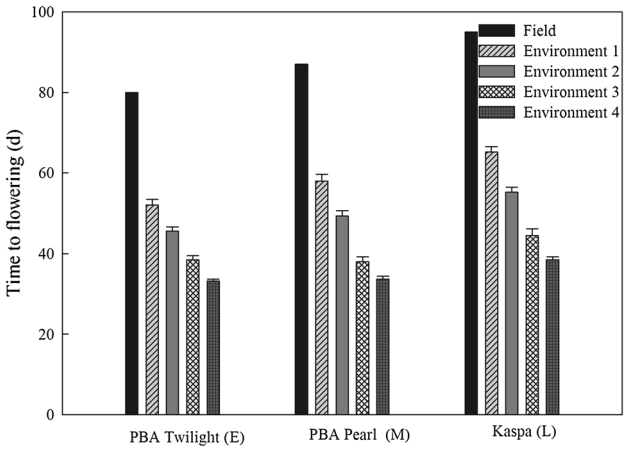

Fig. 1 The effect of environment (Env.) on time to flowering (d) of early (E), mid (M) and late (L) pea flowering genotypes (field data source: Department of Agriculture and Food Western Australia). Data represent mean values ± SE; $n = 10$

subsequent experiments. Growing plants under constant irradiation of 600 µmol m^{-2} s^{-1} (Environment 4) resulted in 4.3–6.0 days faster flowering compared to variable irradiation between 500 and 1000 µmol m^{-2} s^{-1} throughout the day (Environment 3; Table 2). Genotypic differences in time to flowering between early and mid-flowering genotypes observed under a photoperiod of 13–14 h (Environments 1 and 2) disappeared when growing plants under a photoperiod of 20 h (Environments 3 and 4; Fig. 1).

Identification of the earliest embryo developmental stage at which optimal in vitro germination and seedling growth occur

Sucrose concentration and moisture content were measured in immature seeds of PBA Twilight (early), PBA Pearl (mid) and Kaspa (late) produced in Environment 4 (Fig. 2a, b). This analysis was partially repeated in Environment 1 as a control to examine the effect of the environment on sucrose concentration and moisture content. No differences were observed between genotypes in either environment,

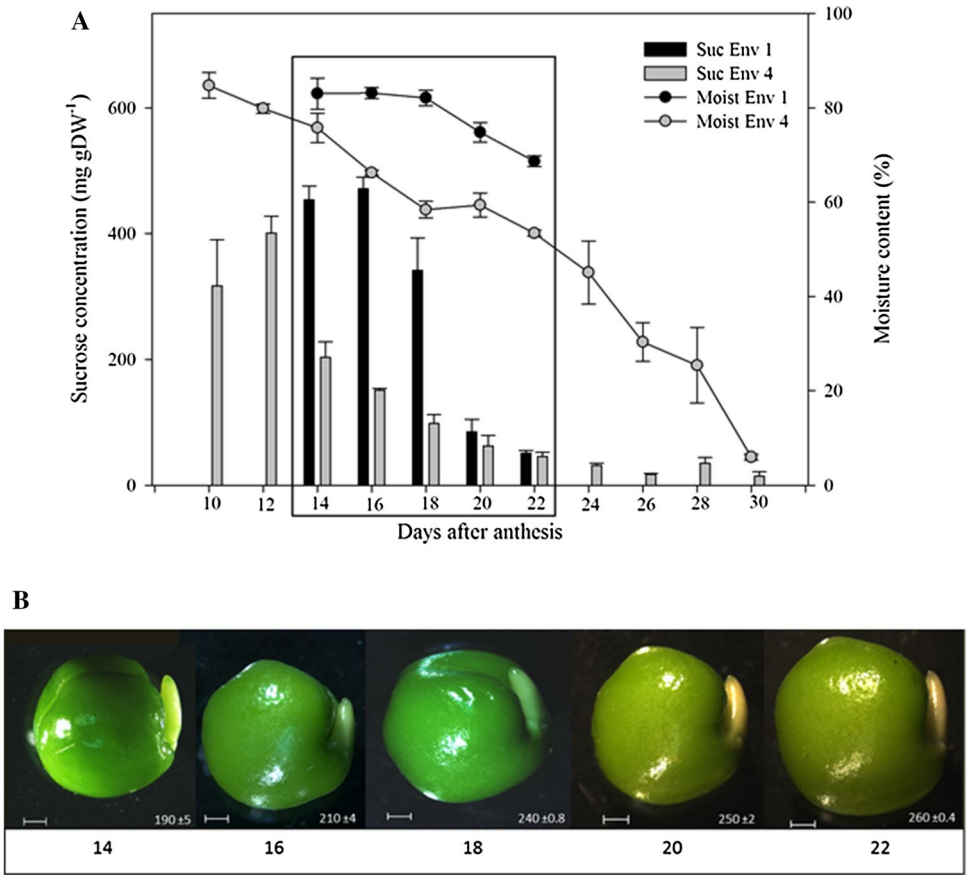

Fig. 2 **a** Sucrose concentration (Suc; mg g^{-1} DW) and moisture content (Moist; %) during pea seed development in Environment 1 (Env 1) and Environment 4 (Env 4). The *box* represents the more narrow range of seed development used to compare between environments. Data represent mean values ± SE; $n = 6$–9. **b** Kaspa seed development and weight (mg; values ± SE; $n = 5$) from 14 to 22 days after pollination in Environment 4 (*scale bar* 1 mm)

therefore, data were combined. The sucrose level in immature seeds was affected by the growing environment ($P \leq 0.05$). The sucrose concentration detected in immature seeds produced in Environment 1 was more than double at 14 DAP and more than three times at 16 and 18 DAP than that observed in Environment 4. Within the time interval studied, a peak in sucrose concentration was observed in Environment 4 in immature seeds between 10 and 12 DAP (300–400 mg g^{-1} DW; Fig. 2a) and Environment 1 between 14 and 16 DAP (450–500 mg g^{-1} DW). An abrupt drop in sucrose levels was observed in Environment 4 between 12 DAP (401 mg g^{-1} DW) and 14 DAP (203 mg g^{-1} DW); while in Environment 1 the sharp drop was delayed by 6 days (from 341 mg g^{-1} DW at 18 DAP to 85 mg g^{-1} DW at 20 DAP). Moisture content in immature seeds in Environment 4 dropped below 60 % between 16 and 18 DAP while in Environment 1 this level presumably occurred sometime after 22 DAP.

In vivo/in vitro accelerated single-seed-descent (aSSD) protocol development

No germination was recorded up to 4 days from in vitro culture for the 14 DAP treatment in any of the tested genotypes (Table 3a). For all genotypes tested, the in vitro culture of immature seeds 18 DAP resulted in higher (PBA Twilight, PBA Hayman, PBA Coogee, OZP1101 and Kaspa) or equal (PBA Pearl, OZP1202 and 00P016-1) percentages of in vitro germination after 4 days of culture compared with the 16 DAP treatment. Variability in plant development was clearly observed between treatments and genotypes 14 days after in vivo transfer (Table 3b). In all genotypes studied, delaying harvest of immature seeds for 2 days, from 16 to 18 DAP, resulted in substantially faster plant development (Table 3b) and flowering (Fig. 3a). The fastest development was observed in genotypes PBA Hayman, PBA Coogee, Kaspa and 00P016-1 where 86–100 % of seedlings originating from the 18 DAP treatment reached, at least, the third-leaf stage 14 days after in vivo transfer. No plants from the 14 DAP treatment reached the third-leaf stage 14 days after in vivo transfer.

Table 3 **a** Percentage of germination 4 days after in vitro culture for 14, 16 and 18 DAP treatments; and **b** Percentage of plants from immature seeds (14, 16 and 18 DAP) that reached, at least, the third-leaf stage 14 days after in vivo transfer

Genotype	a) Germination (%)			b) Plants at the third-leaf stage (%)		
	14 DAP	16 DAP	18 DAP	14 DAP	16 DAP	18 DAP
PBA Twilight (E)	0	81.9	94.0	0	0	39.2*
PBA Pearl (M)	0	99.0	98.0	0	41.4	67.4*
PBA Hayman (L)	0	80.5	100	0	67.5	100*
PBA Coogee (L)	0	93.5	100	0	64.2	100*
OZP1202 (L)	0	100	100	0	0	18.9*
OZP1101 (L)	0	71.0	95.0	0	0	33.7*
Kaspa (L)	0	75.0	100	0	69.3	86.4*
00P016-1 (VL)	0	99.0	100	0	91.7	100

E early flowering, *M* mid flowering, *L* late flowering, *VL* very late flowering

*Indicates significant differences in individual genotypes between 16 and 18 DAP at $P \leq 0.05$; $n = 50$

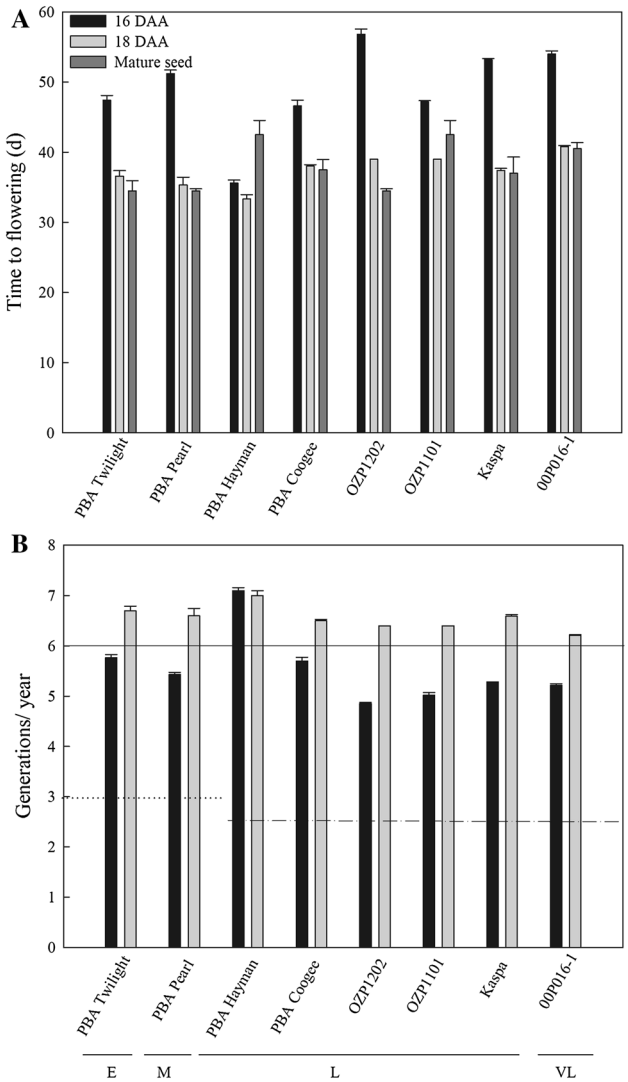

Fig. 3 **a** Time to flowering (d) of in vitro derived plants and mother plants (mature seeds) across genotypes in Environment 4; and **b** Number of generations per year obtained for two different embryo development stages across genotypes (average between two generations). *E* early, *M* mid, *L* late and *VL* very late. *Continuous bar* indicates the number of generations per year that can be obtained with the proposed protocol compared to the conventional single-seed-descent system for early/mid (*dotted bar*) and late (*dashed bar*) flowering genotypes. Data represent mean values ± SE; $n = 10$. The absence of an *error bar* indicates SE = 0

With time to flowering, no delay was observed in any tested genotype (except for OZP1202) by the culture of immature seeds 18 DAP compared to plants originating from mature seeds (Fig. 3a). In genotypes Hayman and OZP1101, floral onset occurred faster with the 18 DAP treatment compared with the culture of mature seeds (between 3.5 and 9 days, $P \leq 0.05$). The 16 DAP treatment delayed flowering time by 7–17 days across genotypes compared with the 18 DAP treatment. Growing plants under our most appropriate in vivo conditions for rapid floral initiation combined with in vitro culture of immature seeds 18 DAP enabled the production of more than six generations per year across the diverse genotypes studied (Fig. 3b).

Discussion

Manipulation of key in vivo growing conditions, including photoperiod, light and temperature, combined with in vitro germination of embryos at a precise developmental stage substantially shortened the life cycle in pea. Seed moisture and sucrose content were confirmed as reliable indicators for the identification of the exact stage for immature seed culture (embryo physiological maturity). For the first time, we have demonstrated that, while it is possible to germinate immature pea seed as early as 14 DAP without exogenous application of hormones, it is more efficient to allow embryos to develop until 18 DAP in terms of generation turnover time. With the proposed protocol, a 60-day generation cycle can now be reliably achieved across phenotypically and genetically-diverse pea germplasm.

The timing of flowering, and, in particular, the degree to which it is responsive to the environment, is a vital element in the adaptation of plant species. Not surprisingly, temperature and photoperiod are key factors in the transition to flowering in legumes (Nelson et al. 2010; Iannucci et al. 2008; Summerfield et al. 1985; Valipour et al. 2015). The effects of light quality on plant growth and floral initiation have been clearly established in long-day plants (Runkle and Heins 2001; Cerdan and Chory 2003). We know that the R:FR ratio is important in floral initiation and the natural R:FR ratio (close to 1:1) seems to be the most effective for flower induction in long-day pea plants (Cummings et al. 2007), so this ratio was closely mimicked in our artificial growth conditions (Environment 4). The generation acceleration protocol presented here has overcome the recalcitrance of very late flowering genotypes to the acceleration of floral initiation. This was an unresolved problem in the four previous reports proposing in vitro based or assisted systems for accelerating generation turnover in pea (Ochatt et al. 2002; Ribalta et al. 2014; Franklin et al. 2000; Ochatt and Sangwan 2010). In this study, a growth temperature of 24/20 °C (day/night) with a photoperiod of 20 h from a combination of metal halide lamps and incandescent globes with a constant irradiance of 600 µmol m^{-2} s^{-1} (Environment 4) provided the most rapid and uniform floral onset and seed set across genotypes, including early, mid, late and very late flowering types. Growing plants under our optimised conditions allowed up to 2.5 times faster floral onset compared to field conditions and up to 1.7 times compared to the conventional SSD system (Environment 1) across the entire range of pea genotypes tested. In long-day plants, longer photoperiods promote earlier flowering. However, substantial variation in the plasticity of this response can be expected among diverse genotypes (Sultan 2000). In our study, later-flowering genotypes showed significant flowering time plasticity mainly in response to photoperiod. For example, growing plants under a 20 h photoperiod (Environments 3 and 4) eliminated differences in time to flowering between early and mid-flowering genotypes that were observed under a 13–14 h photoperiod (Environments 1 and 2; Fig. 1). Also, all the late flowering genotypes we tested behaved as early/mid-flowering types under our optimised conditions (Environment 4; Fig. 3a). This supports the idea presented by Zhang and Lechowicz (1994) that selection for later-flowering genotypes would select concomitantly for greater plasticity.

The success of germination and early plant growth is largely determined by the physiological, genetic and biochemical features of the seed (Gallardo et al. 2008; Hay et al. 2010; Udomdee et al. 2014). A complex regulatory network triggers the initiation of maturation and accumulation of storage products (starch and other carbohydrates) caused by sucrose cleavage (Weber et al. 2005). The seed carbohydrate state provides signals to adjust metabolism to specific physiological conditions. For example, in *Vicia*, high levels of sucrose stimulate starch accumulation in developing cotyledons which are correlated with the onset of embryo maturation (Weber et al. 1998). In pea, seed filling and maturation are linked to a slight decrease in moisture content and are terminated when seed moisture content is close to 55 % (Le Deunff and Rachidian 1988). In our research, we observed clear differences in embryo development between Environments 1 and 4. We estimate that the onset of embryo physiological maturity (when sucrose peak is observed) is achieved 4 days earlier in Environment 4 (between 10 and 12 DAP) compared to Environment 1 (between 14 and 16 DAP). Embryo physiological maturity was fully attained at approximately 18 DAP in Environment 4 and more than 22 DAP in Environment 1, when moisture content was under 60 % and sucrose concentration below 100 mg g^{-1} DW (Fig. 2a). Growing plants in Environment 4 not only resulted in faster floral onset but also accelerated embryo development compared to Environment 1.

According to Gallardo et al. (2003), *Medicago truncatula* seeds acquire the ability to germinate at approximately

14 DAP. In our experiment, seeds harvested 14 DAP had the capacity to germinate in vitro with no requirement for plant growth regulators in the culture medium, although at a much lower rate. Allowing the embryo to mature longer on the plant, and harvesting immature seeds 18 DAP instead of 16 DAP, enabled more robust in vitro germination, faster seedling development and accelerated floral onset in vivo. There is no clarity around early vigour and later developmental penalties that may occur when embryos are precociously germinated. Interestingly, no delay penalty in terms of time to flowering and plant development was caused by the culture of immature seeds 18 DAP compared to the use of mature ones in the tested genotypes (except for OZP1202). The plants produced with the proposed system were morphologically normal and set flowers and normal pods/seed when grown under conventional glasshouse conditions. Achieving embryo physiological maturity (18 DAP in Environment 4) appears to be a pre-requisite for optimal in vitro germination and seedling development from immature seeds. This is in line with recent findings regarding the genetic determinism of embryo development and seed filling, which show that several key transcription factors/genes involved in both these processes are overexpressed at precisely that developmental stage (Noguero et al. 2013). Endoreduplication, the cytogenetic imprint of the onset of the seed-filling phase required for seed germination, also occurs at that stage (Ochatt 2015). Further research is necessary to elucidate the role hormones may play on the acquisition of germination competence during seed development.

The enhanced in vivo/in vitro SSD protocol presented here overcomes previously reported constraints of genotype dependence resulting in a robust, cost-effective and broadly applicable system. This protocol enabled a drastic reduction of the generation cycle in pea compared to the conventional SSD system (Environment 4 vs. Environment 1) by: (1) accelerating floral onset (saving between 20 and 27 days) and (2) reducing the time to harvesting through culture of immature seeds (18 DAP) instead of fully mature seed (45 DAP). This resulted in an overall saving of 47–54 days per generation across genotypes. The use of this technology will enable the development of near-homozygous lines in a single year providing a major advantage to breeding programs for the development of novel genotypes with improved yield and stress resistance. It is clearly also beneficial for the rapid development of recombinant inbred lines (RILs) to map gene-trait associations. The concurrent availability of marker technology and this step reduction in generation cycle time provides breeders with the opportunity to re-design their approach to crop improvement; for example, to rapidly develop complex populations such as multi-parental advanced generation intercrosses (MAGIC) (Cavanagh et al. 2008).

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References

Brim CA (1966) A modified pedigree method of selection in soybeans. Crop Sci 6:220

Cavanagh C, Morell M, Mackay I, Powell W (2008) From mutations to MAGIC: resources for gene discovery, validation and delivery in crop plants. Curr Opin Plant Biol 11:215–221

Cerdan PD, Chory J (2003) Regulation of flowering time by light quality. Nature 423:881–885

Croser JS, Lulsdorf MM, Davies PA, Clarke HJ, Bayliss KL, Mallikarjuna N, Siddique KHM (2006) Toward doubled haploid production in the *Fabaceae*: progress, constraints, and opportunities. Crit Rev Plant Sci 25:139–157

Cummings IG, Reid JB, Koutoulis A (2007) Red to far-red ratio correction in plant growth chambers—growth responses and influence of thermal load on garden pea. Physiol Plantarum 131:171–179

Ellis RH, Hong TD, Roberts EH (1987) The development of desiccation-tolerance and maximum seed quality during seed maturation in six grain legumes. Ann Bot 59:23–29

Franklin G, Pius PK, Ignacimuthu S (2000) Factors affecting in vitro flowering and fruiting of green pea (*Pisum sativum* L.) Euphytica 115:65–74

Gallardo K, Le Signor C, Vandekerckhove J, Thompson RD, Burstin J (2003) Proteomics of *Medicago truncatula* seed development establishes the time frame of diverse metabolic processes related to reserve accumulation. Plant Physiol 133:664–682

Gallardo K, Thompson R, Burstin J (2008) Reserve accumulation in legume seeds. CR Biol 331:755–762

Germanà MA (2011) Anther culture for haploid and doubled haploid production. Plant Cell Tiss Org Cult 104:283–300

Goulden CH (1939) Problems in plant selection. In: Burnett RC (ed) Proceeding of the Seventh Genetic Congress. Cambridge University Press, England, pp 132–133

Hay FR, Smith RD, Ellis RH, Butler LH (2010) Developmental changes in the germinability, desiccation tolerance, hardseededness, and longevity of individual seeds of *Trifolium ambiguum*. Ann Bot 105:1035–1052

Iannucci A, Terribile MR, Martiniello P (2008) Effects of temperature and photoperiod on flowering time of forage legumes in a Mediterranean environment. Field Crop Res 1006:156–162

Le Deunff Y, Rachidian Z (1988) Interruption of water delivery at physiological maturity is essential for seed development, germination and seedling growth in pea (*Pisum sativum* L.) J Exp Bot 39:1221–1230

Maluszynski M, Kasha KJ, Szarejko I (2003) Published doubled haploid protocols in plant species. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled haploid production in crop plants. A manual. Kluwer, Dordrecht, pp 309–335

Mason MG, Ross JJ, Babst BA, Wienclaw BN, Beveridge CA (2014) Sugar demand, not auxin, is the initial regulator of apical dominance. Proc Natl Acad Sci 111:6092–6097

Moe R, Heins R (1990) Control of plant morphogenesis and flowering by light quality and temperature. Acta Hortic 272:81–89

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plantarum 15:473–497

Murfet IC, Reid JB (1974) Flowering in *Pisum*: the influence of photoperiod and vernalising temperatures on the expression of genes *Lf* and *Sn*. Z Pflanzenphysiol 71:323–331

Nelson MN, Berger JD, Erskine W (2010) Flowering time control in annual legumes: prospects in a changing global climate. CAB Rev: Perspect Agr Vet Sci Nutr Natur Resour 5:49–62

Noguero M, Atif RM, Ochatt S, Thompson RD (2013) The role of the DNA-binding One Zinc Finger (DOF) transcription factor family in plants. Plant Sci 209:32–45

Ochatt SJ (2015) Agroecological impact of an in vitro biotechnology approach of embryo development and seed filling in legumes. Agron Sustain Dev 35:535–552

Ochatt SJ, Sangwan RS (2010) In vitro flowering and seed set: acceleration of generation cycles. In: Davey MR, Anthony P (eds) Plant cell culture: essential methods. John Wiley & Sons, Ltd., Chichester, pp 97–110

Ochatt SJ, Sangwan RS, Marget P, Ndong YA, Rancillac M, Perney P (2002) New approaches towards the shortening of generation cycles for faster breeding of protein legumes. Plant Breeding 121:436–440

Redden B, Leonforte T, Ford R, Croser J, Slattery J (2005) Pea (*Pisum sativum* L.). In: Singh RJ, Jauhar PP (eds) Genetic resources, chromosome engineering, and crop improvement, vol 1. Taylor & Francis, Boca Raton, pp 49–83

Ribalta FM, Croser JS, Erskine W, Finnegan PM, Lulsdorf MM, Ochatt SJ (2014) Antigibberellin-induced reduction of internode length favors in vitro flowering and seed-set in different pea genotypes. Biol Plant 58:39–46

Runkle ES, Heins RD (2001) Specific functions of red, far red, and blue light in flowering and stem extension of long-day plants. J Amer Soc Hort Sci 126:275–282

Spalding EP, Folta KM (2005) Illumination topics in plant photobiology. Plant Cell Environ 28:39–53

Sultan SE (2000) Phenotypic plasticity for plant development, function and life history. Trends Plant Sci 5:537–542

Summerfield RJ, Roberts EH, Erskine W, Ellis RH (1985) Effects of temperature and photoperiod on flowering in lentils (*Lens culinaris* Medic.) Ann Bot 56:659–671

Udomdee W, Wen PJ, Lee CY, Chin SW, Chen FC (2014) Effect of sucrose concentration and seed maturity on in vitro germination of *Dendrobium* nobile hybrids. Plant Growth Regul 72:249–255

Vadez V, Berger JD, Warkentin T, Asseng S, Ratnakumar P, Rao KP, Gaur P, Munier-Jolain N, Larmure A, Voisin A-S, Sharma H, Pande S, Sharma M, Krishnamurthy L, Zaman M (2012) Adaptation of grain legumes to climate change: a review. Agron Sustain Dev 32:31–44

Valipour M, Ahmadi MZ, Raeini-Sarjaz M, Sefidkouhi MAG, Shahnazari A, Fazlola R, Darzi-Naftchali A (2015) Agricultural water management in the world during past half century. Arc Agron Soil Sci 61:657–678

Vince-Prue D (1981) Daylight and photoperiodism. In: Smith H (ed) Plants and the daylight spectrum. Academic Press Inc., London, pp 223–242

Weber H, Heim U, Golombek S, Borisjuk L, Manteuffel R, Wobus U (1998) Expression of a yeast-derived invertase in developing cotyledons of *Vicia narbonensis* alters the carbohydrate state and affects storage functions. Plant J 16:163–172

Weber H, Borisjuk L, Wobus U (2005) Molecular physiology of legume seed development. Annu Rev Plant Biol 56:253–279

Weller JL, Murfet IC, Reid JB (1997) Pea mutants with reduced sensitivity to far-red light define an important role for phytochrome A in day-length detection. Plant Physiol 114:1225–1236

Zhang J, Lechowicz MJ (1994) Correlation between time of fowering and phenotypic plasticity in *Arabidopsis thaliana (Brassicaceae)*. Am J Bot 81:1336–1342

Zhou Y, Singh BR (2002) Red light stimulates flowering and anthocyanin biosynthesis in American cranberry. Plant Growth Regul 38:165–171