The Determination Time of the Carpel Whorl Is Differentially Sensitive to Carbohydrate Supply in *Pharbitis nil*[1]

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A shoot apical meristem is florally determined if, following its removal from an induced plant, it flowers when cultured in non-inductive conditions. Determination times were measured in the short-day plant *Pharbitis nil* to examine whether floral whorls are determined simultaneously or sequentially. Shoot apices were excised at daily intervals following a 48-h dark-inductive treatment, cultured in non-inductive conditions for 4 weeks in continuous light, and the number of floral organs scored. The culture medium was White's supplemented with sucrose, glucose (Glc), fructose (Fru), or 1:1 Glc:Fru at 2% (w/v), 4% (w/v), or 6% (w/v) or sugar-mannitol combinations of osmotic potentials equivalent to 4% (w/v) or 6% (w/v). The minimum whorl determination time was 1 d for sepals, petals, and stamens regardless of carbon supply. However, for carpels it varied remarkably from 5 d on sucrose, to 2 to 3 d on Fru or Glc:Fru, to 1 d for 2% (w/v) and 6% (w/v) Glc. Therefore, depending on the carbon supply, the carpel whorl was determined at the same time or after the outer whorls. Generally, these effects could not be reproduced on the sugar-mannitol treatments.

Shoot apical meristems are florally determined when they form flowers in an environment in which vegetative growth would be predicted (McDaniel, 1978; Singer and McDaniel, 1986). Determination is an operational term used in a number of developmental studies. Cells are determined when they have the same developmental fate whether left in situ or isolated from the rest of the organism (McDaniel, 1984). An established way to test for floral determination is to provide a potentially inductive treatment, culture the shoot apex under non-inductive conditions, and record whether it forms a flower in vitro (McDaniel et al., 1991).

An unresolved question is whether floral whorls are determined simultaneously, in an all-or-nothing response, or independently of each other both in space and time. In the long-day (LD) plant *Lolium temulentum*, cultured apices formed flowers when they were removed from the plant 22 h after the start of the inductive LD but not before (McDaniel et al., 1991). The authors concluded that all whorls were determined simultaneously (McDaniel et al., 1991). Floral determination was also examined in another LD plant, *Silene coeli-rosa*, which requires 7 LD for 100% flowering. However, 4 LD is a threshold inductive treatment resulting in 20% to 40% of flowering (Donnison and Francis, 1993). Excising apices from plants that received 4 LD resulted in 15% of the apices forming sepals in vitro, but 6 LD were necessary for 50% of them to form sepals, so an estimate for determination of the sepal whorl was 2 d (6 − 4 LD). Seven LD were necessary before 50% of apices exhibited stamens and petals, while 7 LD + 1 short d (SD) were required for the carpels. Therefore, the stronger the inductive treatment, the more floral whorls in vitro (Donnison and Francis, 1993, 1994). Overall, these data are consistent with a sequential determination of each whorl, as opposed to an all-or-nothing determination of the entire apex. The determination and initiation of the carpel whorl began after the other whorls were determined and initiated, so that the operational definition of determination was refined to account for the determination of each whorl (Donnison and Francis, 1993).

To our knowledge, floral determination times have been tested for only one SD plant, *Pharbitis nil.* The reported determination times for *P. nil* vary from 5 h (Matsushima et al., 1974), to 9 h (Larkin et al., 1990), to 36 h (Bhar, 1970). The variable times reflect the different methods chosen: Bhar (1970) and Matsushima et al. (1974) used tissue culture, while Larkin et al. (1990) used grafting. We also wondered about the effectiveness of the single 16-h inductive dark period used in these studies, particularly since in our study 24 h of darkness was insufficient for 100% flowering of the terminal and axillary meristems (Herbert et al., 1992)—48 h of darkness was necessary before all terminal shoot apical meristems were induced to flower (Herbert et al., 1992; Durdan et al., 1998).

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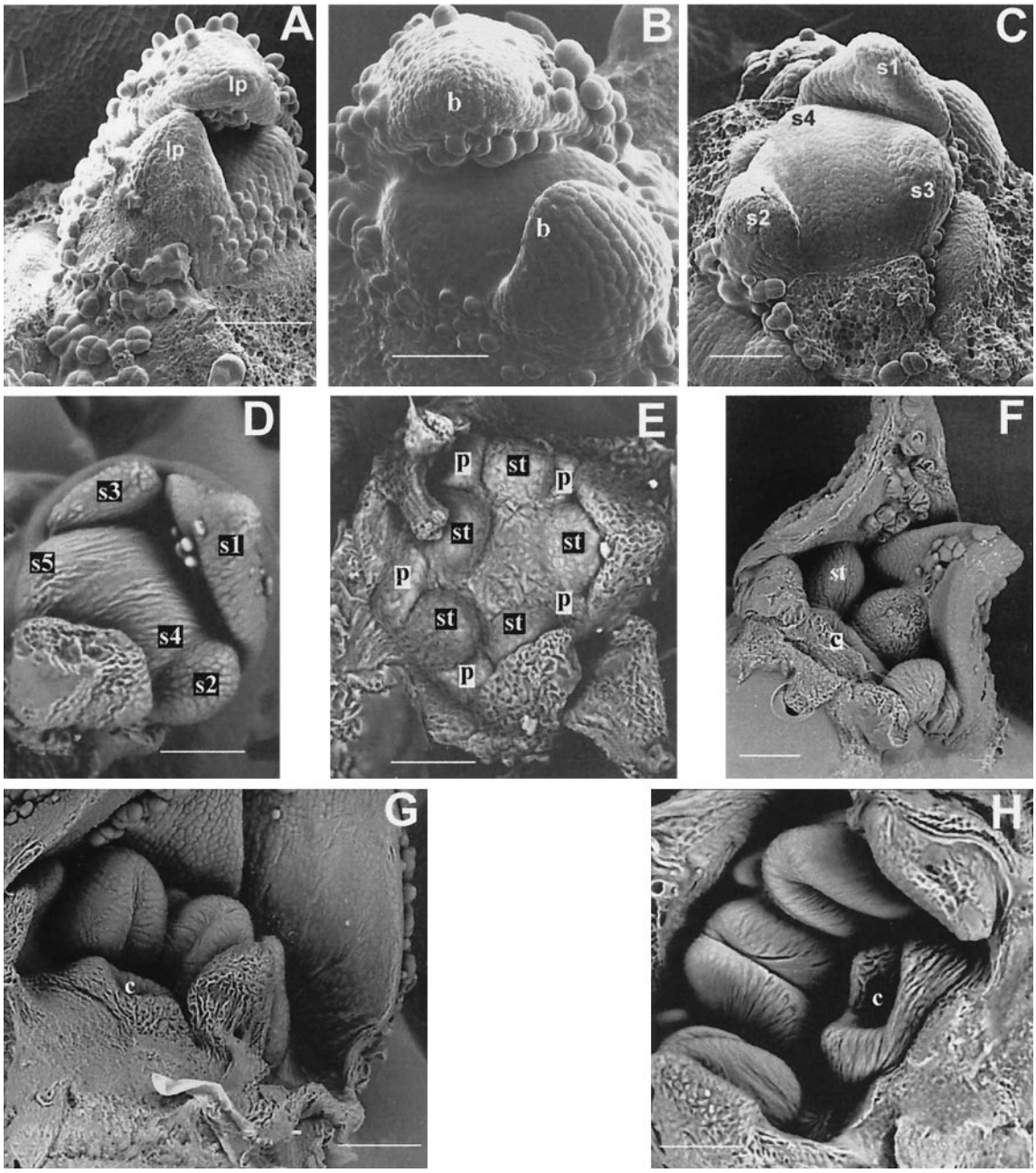

Figure 1. Composite of low-vacuum scanning electron photomicrographs of shoot apical meristems of *P. nil* undergoing the floral transition at various times following induction of 5-d-old seedlings with 48 h of darkness (bar scale = 100 μm). A, 1-d apex with vegetative leaf primordia (lp); B, 2-d apex which with two bract primordia (b) directly opposite each other; C, 3-d apex with sepal primordia forming (s1–4); D, 4-d apex showing all five sepal primordia (s1–5) surrounding the apex. A partially dissected leaf primordium remains on this specimen, but the bracts were removed prior to embedding; E, 5-d apex with partially dissected mature sepals surrounding a whorl of five petal primordia (p) alternating with five stamen primordia (st); F, 6-d apex showing two sepals and four stamens (st) to one side of the carpels (c). The latter are visible for the first time as a small raised ring in the center of the apex. The other organs of the earlier whorls were dissected prior to embedding; G, 7-d apex showing further development of the carpel primordia (c), which are now visible as three distinctive peaks at the center of the apex. Surrounding the carpel primordia are stamens, petals, and sepals, but some of the organs of earlier whorls are dissected; H, 8-d apex with three large, distinctive carpel (c) primordia fused together to form a cone-shaped gynoecium. The stamen, petal, and sepal whorls surrounding the carpel primordia are prominent, but some organs are dissected in this preparation.

Given the variable results obtained previously, we decided to re-investigate floral determination times for *P. nil* using our strong inductive treatment. The aim of the work reported here was to examine whether floral determination is an all-or-nothing phenomenon in the SD plant *P. nil*, as reported for

Table I. *Numerical scoring scheme of floral development in the terminal apex of P. nil*

The system is a progressive one so that each number represents an additional stage of floral morphogenesis. The day of appearance following induction of each whorl in vivo is also indicated.

Score	Description of Development in the Terminal Apex	Day of Appearance
0	Vegetative apex (Fig. 1A)	
1	Formation of the two floral bracts (Fig. 1B)	2
2	Formation of less than five sepal primordia (Fig. 1C)	3
3	Formation of all five sepal primordia (Fig. 1D)	
4	Development of five distinct sepals	
5	Formation of less than five petal and stamen primordia	5
6	Formation of all five petal and stamen primordia (Fig. 1E)	
7	Development of the five petal and stamens	
8	Initial formation of the carpel whorl-ring structure (Fig. 1F)	6
9	Development of the carpels, now visible as three peaks (Fig. 1G)	
10	Further development of the carpels, now visible as a cone-like structure (Fig. 1H)	

the LD plant *L. temulentum* (McDaniel et al., 1991), or whether it was sequential, whorl by whorl, as in the LD plant *S. coeli-rosa* (Donnison and Francis, 1993). Determination times of the three outer whorls were coincident and largely unaffected by carbon supply, but carpel determination time was remarkably variable—3 to 5 d on Suc but 1 d on Glc—leading us to propose a role for this monosaccharide in flowering.

RESULTS

Floral Scoring Scheme

P. nil apical meristems were observed by scanning electron microscopy throughout floral initiation. To our knowledge, this is the first pictorial record of floral development in *P. nil* following a strong 48-h inductive treatment. On d 1, the shoot apical meristem appeared vegetative (Fig. 1A), and the first stage of floral morphogenesis, bract development, was not observed until d 2 (Fig. 1B). By d 3, sepal primordia were present and the apex appeared as a broad smooth dome (Fig. 1C). On d 4, all five sepals were apparent (Fig. 1D). One day later, alternating petal and stamen primordia were observed (Fig. 1E), which by d 6 became more distinctive from each other (Fig. 1F). At that time, the sepals were fully developed, and, a raised ring was observed comprising the carpel primordia at the center of the widening apex (Fig. 1, F–G). On d 7, the carpel primordia were clearly visible as three distinctive peaks at the center of the apex (Fig. 1G), which by d 8 resembled a cone-like structure (Fig. 1H).

Observations were grouped into three classes: (a) appearance of the first primordia, (b) appearance of all primordia in the whorl, and (c) enlargement (maturation) of all organs in the whorl. This led to a scoring system based on three successively higher scores per whorl, with the petal and stamen whorls grouped together because they were initiated and developed together (Fig. 1E). This scheme, similar to that of Maeska and Ogawa (1994) using a 16-h inductive dark treatment, was used to score floral morphogenesis of *P. nil* in vitro (Table I).

Cotyledon Removal

When the cotyledons were removed <16 h after the start of the 48-h inductive dark period flowers began to form at the terminal apex, but 48 h of darkness was necessary for 100% terminal flowers (Table II). The data are consistent in showing that under the conditions employed in our experiments, the floral stimulus began to move from the cotyledons between 12 and 16 h, but 48 h of darkness were necessary for all terminal apices to become floral.

Floral Determination

Floral determination was examined by culturing apices at various times following induction on media supplemented with qualitatively different carbohydrate sources and scoring them 4 weeks later, as explained above. Because all observations were of primordia in whorls, we measured whorl determination times in two ways. In the first method, times

Table II. *Percentage flowering at the shoot apical meristem when the cotyledons were removed at various times (h) following the start of a 48-h inductive dark treatment and the plants were returned to continuous light and scored 4 weeks later (n = 12)*

Time of Cotyledon Removal	Percentage Flowering
h	
0	0
4	0
8	0
12	0
16	83
20	92
24	92
48	100

Table III. *The percentage of apices that formed sepals (s); sepals, petals, and stamens (s/ps); or sepals, petals, stamens, and carpels (s/ps/c) upon examination 4 weeks following their removal and culture on basal medium supplemented with Suc, Glc, Fru, or 1:1 Glc:Fru: 2% (w/v) (A), 4% (w/v) (B), 6% (w/v) (C) or sugar-mannitol combinations that generated osmotic potentials equivalent to 4% (D) or 6% (E) Suc, Glc or Fru*

The percentage data are derived from accumulated scores within days. For example, 6 d following induction on 2% (w/v) Suc (Fig. 2A), 81% of apices achieved a floral score of carpels (22/27), while 11% (3/27) were petals and stamens, and 8% (2/27) were sepals only. However, to achieve these scores, 100% (27/27) exhibited sepals (100%) and 93% (25/27) exhibited stamens and petals. *n* values for Suc ≤ 19 ≥ 30 (2%), ≤ 14 ≥ 19 (4%), ≤ 10 ≥ 17 (6%); Suc:mannitol ≤ 6 ≥ 12 (4%), ≤ 6 ≥ 15 (6%); Glc ≤ 21 ≥ 32 (2%), ≤ 10 ≥ 12 (4%), ≤ 10 ≥ 14 (6%); Glc-mannitol, ≤ 7 ≥ 12 (4%), ≤ 5 ≥ 13 (6%); Fru ≤ 18 ≥ 26 (2%), ≤ 12 ≥ 16 (4%), ≤ 14 ≥ 18 (6%); Fru-mannitol ≤ 9 ≥ 23 (4%), ≤ 10 ≥ 11 (6%); Glc:Fru ≤ 10 ≥ 16 (2%), ≤ 10 ≥ 24 (4%), ≤ 11 ≥ 20 (6%).

	Days following Induction						Days following Induction			
	1	2	3	4	5	6	1	2	3	6
2% Suc (Fig. 2A)										
s	100	100	100	100	100	100				
s/ps	20	9	45	100	100	93				
s/ps/c	0	0	0	0	63	81				
4% Suc (Fig. 2B)							Suc-mannitol ≡ 4% Suc (Fig. 2D)			
s	100	100	100	–	–	100	100	100	100	100
s/ps	33	56	89	–	–	93	0	10	82	86
s/ps/c	0	0	32	–	–	93	0	0	0	86
6% Suc (Fig. 2C)							Suc-mannitol ≡ 6% Suc (Fig. 2E)			
s	100	100	100	–	–	100	100	100	100	100
s/ps	27	90	100	–	–	94	0	0	0	100
s/ps/c	0	0	59	–	–	82	0	0	0	94
2% Glc (Fig. 6A)										
s	100	100	100	–	–	100				
s/ps	37	10	52	–	–	65				
s/ps/c	20	10	23	–	–	65				
4% Glc (Fig. 6B)							Glc-mannitol ≡ 4% Glc (Fig. 6D)			
s	100	100	100	–	–	100	100	100	100	100
s/ps	25	53	83	–	–	90	0	0	36	83
s/ps/c	0	0	17	–	–	80	0	0	0	58
6% Glc (Fig. 6C)							Glc-mannitol ≡ 6% Glc (Fig. 6E)			
s	100	100	100	–	–	100	100	100	100	100
s/ps	20	50	100	–	–	100	0	10	0	60
s/ps/c	20	6	67	–	–	100	0	10	0	40
2% Fru (Fig. 7A)										
s	100	100	100	–	–	100				
s/ps	12	17	90	–	–	94				
s/ps/c	0	0	57	–	–	89				
4% Fru (Fig. 7B)							Fru-mannitol ≡ 4% Fru (Fig. 7D)			
s	100	100	100	–	–	100	100	100	100	100
s/ps	13	50	92	–	–	100	0	0	22	96
s/ps/c	0	25	58	–	–	87	0	0	0	96
6% Fru (Fig. 7C)							Fru-mannitol ≡ 6% Fru (Fig. 7E)			
s	100	100	100	–	–	100	100	100	100	100
s/ps	0	0	60	–	–	100	0	9	100	100
s/ps/c	0	0	59	–	–	82	0	0	0	92
2% Glc:Fru (Fig. 8A)										
s	100	100	100	–	–	100				
s/ps	9	17	100	–	–	91				
s/ps/c	0	17	100	–	–	91				
4% Glc:Fru (Fig. 8B)										
s	100	100	100	–	–	100				
s/ps	7	29	86	–	–	100				
s/ps/c	0	0	71	–	–	100				
6% Glc:Fru (Fig. 8C)										
s	100	100	100	–	–	100				
s/ps	24	13	67	–	–	87				
s/ps/c	0	0	25	–	–	67				

were estimated as the interval between the end of induction and the day of explantation, which resulted in the first appearance of that whorl when the cultured apices were examined 4 weeks later—the minimum whorl determination time. In other words, this method was a measure of the minimum time that

the apex had to be left on the plant for it to form that whorl in vitro. In the second method, determination times were recorded as the interval between the end

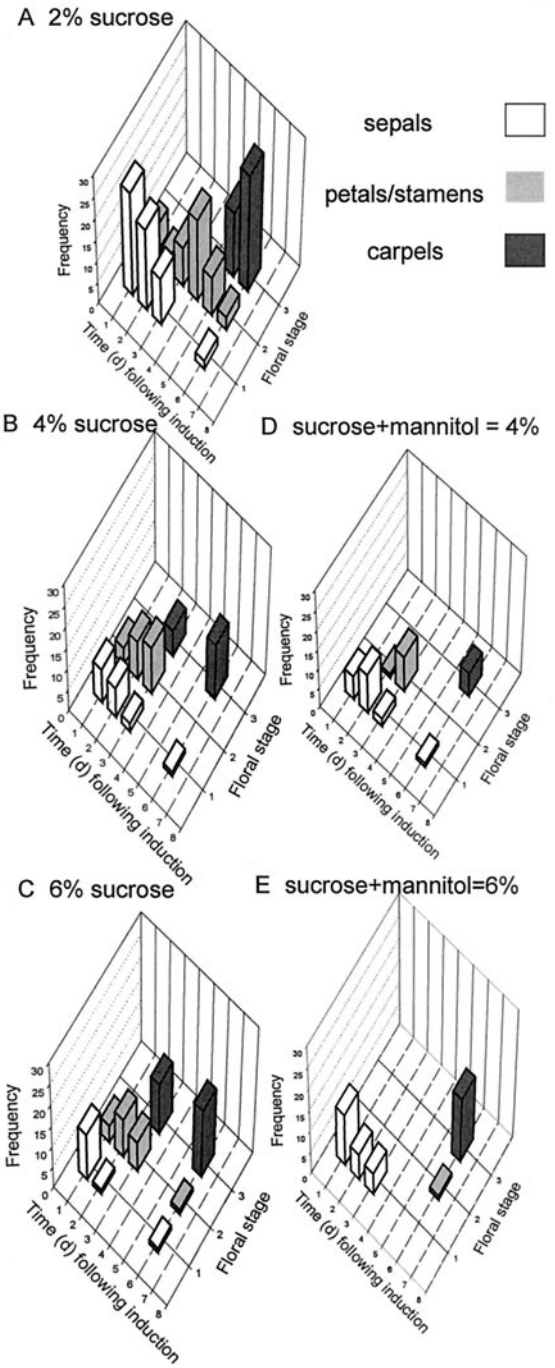

Figure 2. Frequency with which apices exhibited each floral whorl when the apices were removed from the plant at various times following induction and cultured in continuous light for 28 d on White's medium supplemented with Suc: A, 2% (w/v) Suc; B, 4% (w/v) Suc; C, 6% (w/v) Suc; D, Suc-mannitol ≡ 4% (w/v) Suc; E, Suc-mannitol ≡ 6% (w/v) Suc. The *z* axis portrays stages of floral morphogenesis: 1, sepal whorl (scores of 2–4); 2, petal/stamen whorls (scores of 5–7); and 3, carpel whorl (scores of 8–10).

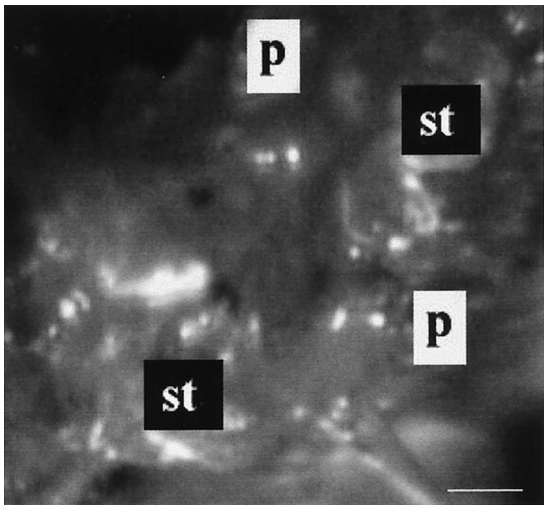

Figure 3. Light microscopic photograph of a shoot apical meristem of *P. nil*, which was removed as part of an apical explant (see "Materials and Methods") from a plant 1 d following a 48-h dark period, and cultured on 2% (w/v) Suc for 5 weeks. This apex exhibited sepals (dissected in this prep), petals (p), stamens (st), and, in the center, a flattened dome but no carpels (bar = 100 μm). There was no evidence of floral reversion. (Consistently, cultured meristems shriveled under low-vacuum scanning electron microscopy.)

of the inductive treatment and the day of explantation, which resulted in ≥50% of apices exhibiting that whorl after 4 weeks in culture, the converse of an LD_{50} (average whorl determination time). However, neither in this nor any comparable study was the operational definition of whorl determination time extended to include the subsequent development of the whorl through to sexual maturity.

The data are presented as a series of three-dimensional plots portraying the frequency of each whorl following explantation on each day of each treatment. For the 2% treatments, they represent pooled data from either two or three replicate experiments. Mann-Whitney tests did not allow rejection of the null hypothesis that there were no significant differences between floral scores within day within treatments ($P \geq 0.6$; $n = 8$–10), indicating the repeatable nature of the observations and justifying our decision to pool data from different experiments. These plots represented a qualitative portrayal of the data, enabling us to identify the first appearance of each whorl. In other words, the three-dimensional figures indicate minimum whorl determination times. In Table III, data are presented as percentages of apices that formed each whorl. However, they are not a reproduction of the three-dimensional plots, because they are derived from accumulated scores within each day. For example, on d 6, 81% (22/27) of apices exhibited carpel whorls, while 11.5% (3/27) exhibited petal/stamen whorls, and 7.5% (2/27) formed the sepal whorl only (Figure 2A). However, to achieve these floral scores, they all formed the sepal whorl (i.e. 100%), 93% (25/27) formed the pet-

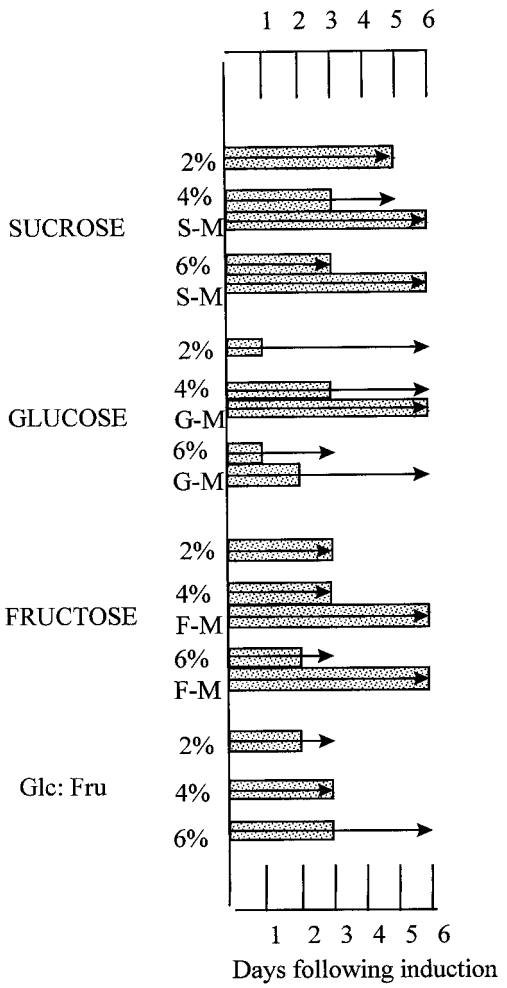

Figure 4. Minimum determination times (dotted rectangles) superimposed on average determination times (arrows) in days for the carpels on 2% (w/v), 4% (w/v), or 6% (w/v) Suc, Glc, Fru, or equimolar Glc:Fru, together with corresponding determination times on sugar-mannitol (−M) combinations that generated osmotic potentials equivalent to the 4% or the 6% carbohydrate.

als/stamen whorls, but only 81% formed the carpel whorls (Table III). Therefore, the tabulated data are a quantitative presentation enabling average determination times (≥50% of apices forming a whorl) to be recorded.

Suc Treatment

When apices were excised 1 d following induction and cultured on 2% (w/v) Suc, 100% (30/30) exhibited sepals, 20% (6/30) formed petals and stamens, but none formed carpels (Fig. 2A; Table III). These apices did not exhibit floral reversion (Fig. 3). Moreover, apices excised 1 d following induction and cultured on 2% (w/v) Suc for 10 weeks did not form carpels (Durdan, 1998). Only when the apex was left on the plant for 5 to 6 d and then cultured did we observe the carpel whorl in vitro (Fig. 2A; Table III). The data are consistent in revealing a minimum whorl determination time of 1 d for sepals, petals, and stamens, but 5 d for carpels (Fig. 4). Note that the lack of carpels in this treatment was not because the explants had stopped growing in vitro (Fig. 5).

On 2% (w/v) Suc, while 20% of apices formed the petals/stamen whorls when the apex was removed 1 d following induction, 45% (9/20) did so at 3 d and 100% at 4 d, resulting in an average determination time of 3 to 4 d for these whorls (Fig. 2A). Increasing the concentration of Suc to 6% (w/v) resulted in the carpel whorl forming in 59% (10/17) of apices that were removed on d 3 (Fig. 2C; Table III). This reduction in average carpel whorl determination time to 3 d was not due to an osmotic effect, because the equivalent combination of Suc and mannitol lengthened the carpel determination time back to 5 to 6 d (Figs. 2E and 4). Both the minimum and average whorl determination times for the petals/stamens were remarkably constant (1 and 2–3 [4] d, respectively) regardless of the amount of Suc added to the medium (non-significant χ^2 [4 df] when the percentage of apices exhibiting petals and stamens were compared between the 2% [w/v], 4% [w/v], and 6% [w/v] Suc treatments). However, the 6% (w/v) Suc-mannitol combination delayed the appearance of the petal/stamen whorls until d 6, perhaps because of a reduction in available carbohydrate (Fig. 2E; Table III).

Glc Treatment

We were surprised to discover that the determination time of the carpel whorl was remarkably sensitive to the type of carbohydrate supplied to the medium. Notably, the 2% (w/v) Glc treatment resulted in the carpel whorl forming in 20% (6/30) of apices that were excised 1 d following induction (Fig. 6A; Table III), an effect reproduced on 6% (w/v) Glc (Fig. 6C) but not on 4% (w/v) Glc (Fig. 6B) or on either of the Glc-mannitol combinations (Fig. 6, D–E; Table III). Therefore, changing the carbon supply from Suc to Glc resulted in a shortening of the minimum carpel whorl determination time from 5 to 1 d (highly significant χ^2 when the percentage of apices forming carpels was compared between the 2% [w/v] Glc and Suc treatments; $P < 0.001$). Note that for the 2% (w/v) and 4% (w/v) Glc treatments, it was not until d 6 that ≥50% of the apices formed the carpel whorl in vitro (Table III), resulting in an average determination time of 6 d (Fig. 4). However, Glc was the only carbon source that resulted in the carpel whorl forming in apices removed 1 d following induction (Fig. 6, A and C).

Another surprising observation was that 10% (1/10) of apices formed carpels on d 2 (minimum determination time of 2 d) on the Glc-mannitol equivalent to 6% (w/v) Glc (Fig. 6E; Table III). This was the only sugar-mannitol treatment to have this effect on carpel whorl determination time. Also, on the 2% (w/v) Glc

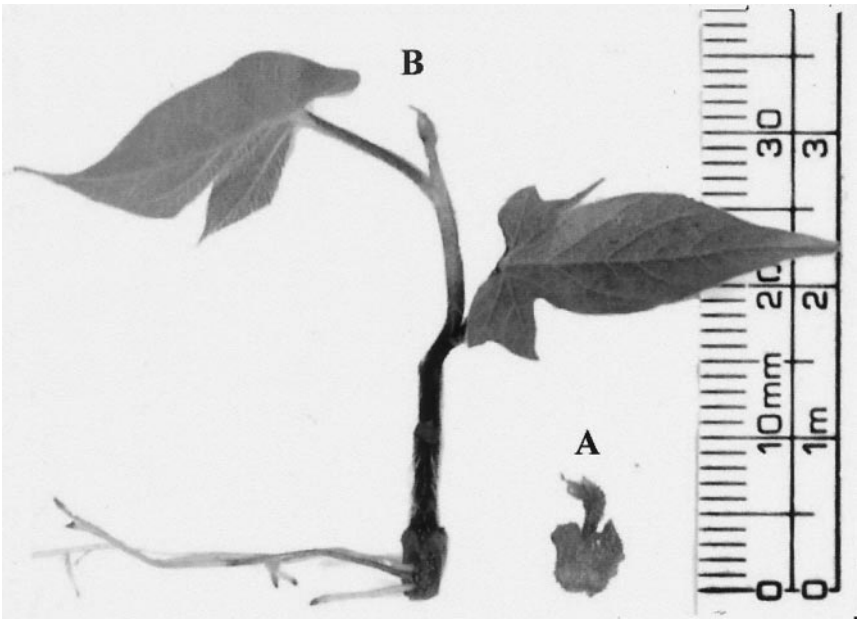

Figure 5. A, An apical explant at the time of culture (1 d following the inductive treatment) alongside B, an explant cultured on 2% (w/v) Suc for 4 weeks showing substantial leaf expansion and obvious root growth.

treatment, over one-third (24/30 = 37%) of the apices formed the petal and stamen whorls 1 d following induction (Table III) resulting in a minimum determination time of 1 d for this whorl. However, the average determination time for these whorls was 2 to 3 d which was generally the case on 2% (w/v), 4% (w/v), and 6% (w/v) Glc (Fig. 6; Table III) illustrating how remarkably stable these whorls were regardless of quantitative changes in the supply of Glc.

Fru and Glc:Fru Treatments

Compared with Suc, the minimum determination time for the carpel whorl was shortened to 2 to 3 d on all three concentrations of Fru (Figs. 4 and 7, A–C). This effect could not be reproduced by the Fru-mannitol combinations, making interpretations based upon osmotic effects unlikely (Fig. 7, D and E). In the Glc: Fru treatments, the minimum carpel whorl determination time was 2 to 3 d (Figs. 4 and 8, A–C), which was the average (Table III) except for 6% (w/v) Glc: Fru, where it was 6 d (Figs. 4 and 8C; Table III).

On both Fru and Glc:Fru, the minimum petal/stamen whorl determination time was 1 d, except on 6% (w/v) Fru, where it was 3 d (Fig. 7C). The average whorl determination time for the petals/stamens in these treatments was 3 d, except for 4% (w/v) Fru, where it was 2 d. These data illustrate the stability of whorl determination times for these whorls regardless of either quantitative or qualitative changes to the carbon supply (Table III). Since shoot apical meristems increase in size to accommodate the floral whorls, we take this as evidence that the growth of the meristems was not substantially different between treatments.

DISCUSSION

In our view, the highlight of the response of shoot apical meristems of induced plants to the different culture media was the remarkable shortening of minimum carpel whorl determination time from 5 d on 2% (w/v) Suc to 3 d both on 6% (w/v) Suc and 2% (w/v) and 6% (w/v) Fru, to 2 d on 4% (w/v) Fru, and down to 1 d on 2% and 6% (w/v) Glc. In order of effectiveness in shortening the minimum whorl determination times, we rank the carbohydrates in the following order: Glc > Fru > Glc:Fru > Suc.

As an example of the way we envisage carbohydrate-mediated regulation of determination times, Figure 9 portrays the floral timetable in vivo (described in Fig. 1 and Table I), together with the minimum determination times for each whorl in the 2% (w/v) Suc and 2% (w/v) Glc treatments. Figure 2A depicts a coincident determination time of 1 d for the sepal, petal, and stamen whorls, but a protracted whorl determination time of 5 d for the carpels in the 2% (w/v) Suc treatment. In our experiments, the floral stimulus began its export from the cotyledons between 12 and 16 h after the start of the inductive treatment (Table II), which is also consistent with the critical dark period of >11 h for this variety of *P. nil* (Vince-Prue and Gressel, 1985). Therefore, an absolute determination time for the outer three whorls could be 56 h (i.e. 16–48 h of the dark period plus the 24 h after induction), which is closer to Bhar's (1970) 36 h than the 5 to 9 h found by Matsushima et al. (1974) and Larkin et al. (1990). In the present study, culturing apices at times within the inductive treatment reduced the overall floral scores, because dark periods <48 h were not sufficient to cause 100% terminal flowering

(Table II). Nevertheless, it is of interest that when apices were removed at times within the dark period and cultured on Suc, some remained vegetative and

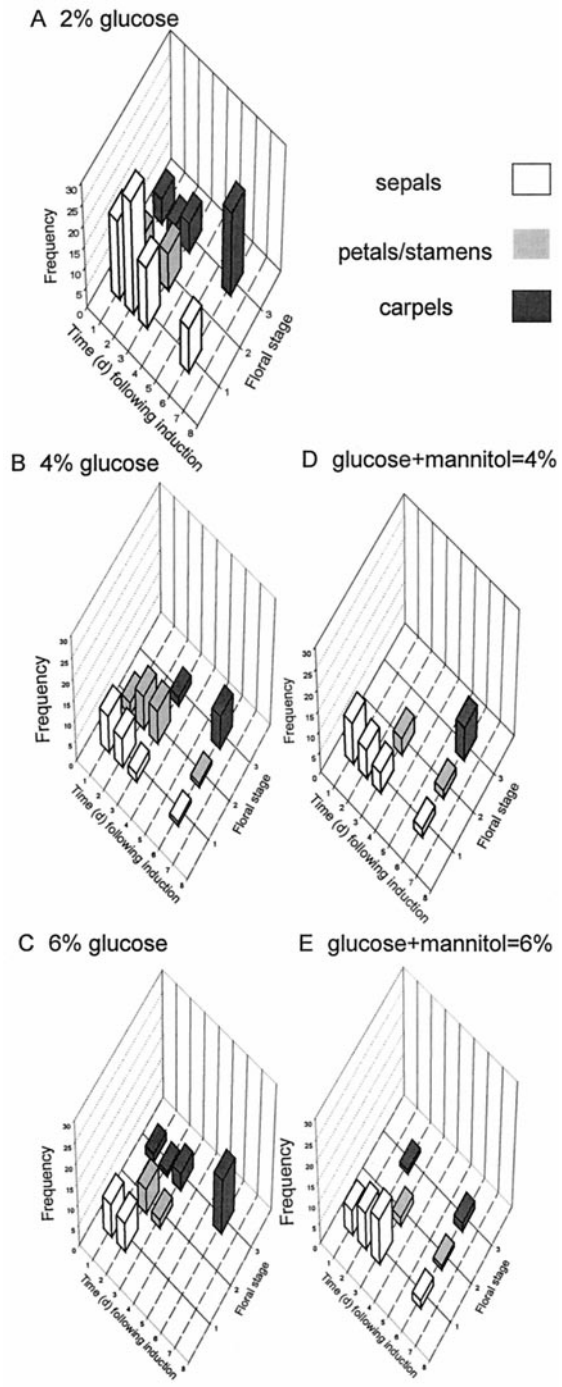

Figure 6. The frequency with which apices exhibited each floral whorl when the apices were removed from the plant at various times following induction and cultured in continuous light for 28 d on White's medium supplemented with Glc: A, 2% (w/v); B, 4% (w/v); C, 6% (w/v); D, Glc-mannitol = 4% (w/v) Glc; E, Glc-mannitol = 6% (w/v) Glc. The *z* axis portrays stages of floral morphogenesis: 1, sepal whorl (scores of 2–4); 2, petal/stamen whorls (scores of 5–7); and 3, carpel whorl (scores of 8–10).

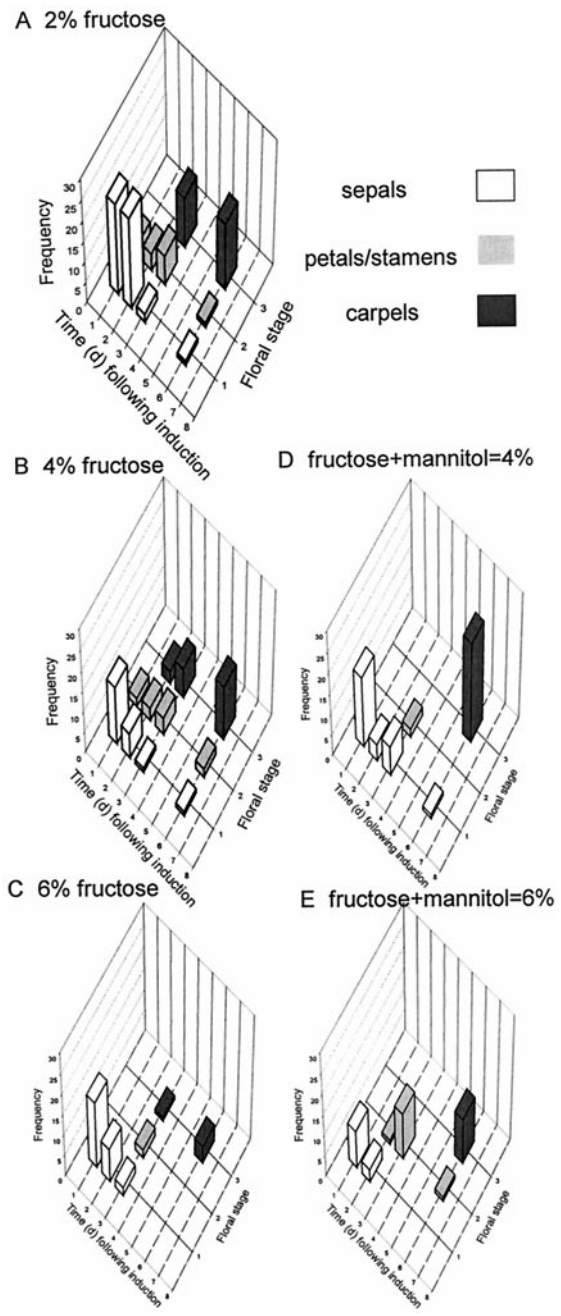

Figure 7. Frequency with which apices exhibited each floral whorl when they were were removed from the plant at various times following induction and cultured in continuous light for 28 d on White's medium supplemented with Fru: A, 2% (w/v); B, 4% (w/v); C, 6% (w/v); D, Fru-mannitol = 4% (w/v) Fru; E, Fru-mannitol = 6% (w/v) Fru. The *z* axis portrays stages of floral morphogenesis: 1, sepal whorl (scores of 2–4); 2, petal/stamen whorls (scores of 5–7), and 3, carpel whorl (scores of 8–10).

some formed the outer three whorls but not the carpel whorl (S. Tudge and R.J. Herbert, unpublished data).

The protracted determination time for the carpel whorl in the 2% (w/v) Suc treatment was not reported previously (Bhar, 1970; Matsushima et al.,

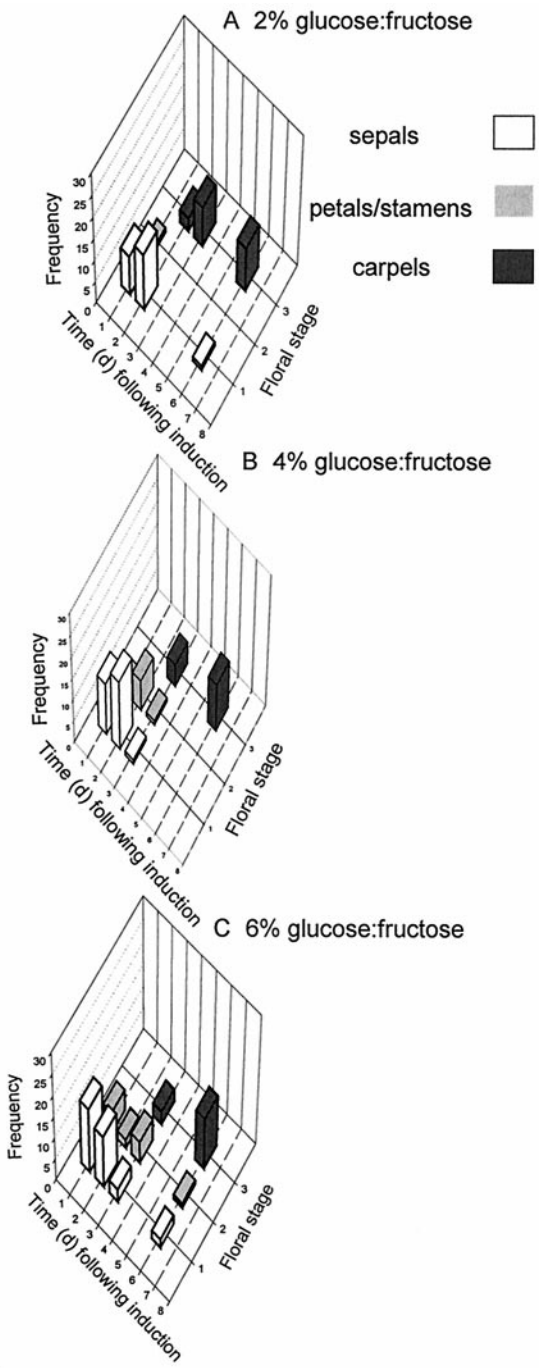

Figure 8. Frequency with which apices exhibited each floral whorl when they were removed from the plant at various times following induction and cultured in continuous light for 28 d on White's medium supplemented with 1:1 Glc:Fru: A, 2% (w/v); B, 4% (w/v); or C, 6% (w/v). The *z* axis portrays stages of floral morphogenesis: 1, sepal whorl (scores of 2–4); 2, petal/stamen whorls (scores of 5–7); and 3, carpel whorl (scores of 8–10).

1974; Larkin et al., 1990). Bhar (1970) cultured the apices on Murashige and Skoog (1962) medium but did not state the amount of Suc added. If it was ≥3%, this would fit with the shorter average determination times we observed at higher Suc concentrations. Larkin et al. (1990) recorded 9 h using grafting. This rapid determination time, using an in vivo approach, is interesting in relation to our Glc results (see below).

Figure 6A also depicts the 2% (w/v) Glc treatment shortening carpel whorl determination time to 1 d, coincident with sepal, petal, and stamen determination. We conclude that this treatment, together with the 6% (w/v) Glc treatment, resulted in a coincident determination time of 1 d for all whorls (Fig. 9B). However, we do not dispute the subjective nature of this interpretation, because, as pointed out above, the average determination time for the carpel whorl varied between 3 and 6 d depending on carbon supply (Fig. 4). Fru and equimolar Glc:Fru also resulted in marked shortening of the minimum carpel whorl determination time to 2 to 3 d, suggesting that genes that regulate determination may be more rapidly expressed in the presence of the monosaccharides than with their parent disaccharide. We also recognize that the 4% (w/v) Glc result is somewhat anomalous in that the minimum determination time lengthened to 3 d for this treatment. Therefore, it is

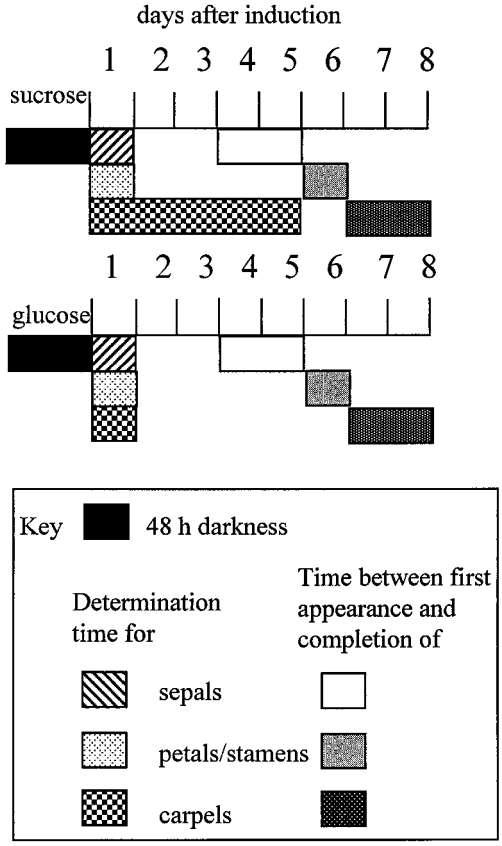

Figure 9. The interval between the first appearance and completion of each whorl in vivo following the 48-h inductive period based upon the scanning electron microphotographs (Fig. 1) and the observations listed in Table I. Minimum determination times (see Figs. 3 and 6) for A, the 2% (w/v) Suc, and B, the 2% (w/v) Glc treatments are displayed as hatched squares/rectangles.

difficult to argue for a concentration-dependent Glc effect. A clearer explanation of this particular result would benefit from greater replication of this treatment.

Suc is the main transport form of assimilated carbon, and is only utilized when cleaved either by invertase or Suc synthase (Copeland, 1990; Avigad and Dey, 1997). Since only Glc treatment caused the dramatic appearance of carpels in meristems cultured 1 d following induction, invertase activity may be more important in the shoot apical meristem in *P. nil* because this enzyme releases Glc, whereas Suc synthase cleaves Suc only in the presence of UDP to form Fru and UDP-Glc (Copeland, 1990). This could have been the case in grafting experiments whereby a rapid release of Glc in the terminal meristem could have resulted in the rapid determination time recorded (Larkin et al., 1990). Invertase activity increased substantially when shoot apical meristems of *Sinapis alba* became committed to flowering (Pryke and Bernier, 1978).

At the molecular level, we suggest that Glc and Fru treatment can shorten carpel determination time by activating the rapid and early expression of homeotic genes involved in carpel development. Lyndon (1998) described a model for determination that involved the binding of homeotic gene products that act as feedback activators of the transcription of downstream homeotic genes (Davies and Schwarz-Sommer, 1994). In this model, determination is the time taken to lock in homeotic gene transcription (Lyndon, 1998).

Carbohydrate-regulated gene expression has already been identified in higher plants with excesses or depletions of sugars, either enhancing or repressing gene expression in a whole range of different processes (e.g. Koch, 1996). Simoko (1994) observed carbohydrate-responsive changes in gene expression of unidentified developmental genes involved with potato tuber induction, which would indicate that sugar-related gene expression may have a major role in changes in plant development. (e.g. Borisjuk et al., 1998). Although Glc-regulated gene expression has been studied in both prokaryotes and eukaryotes, the Glc-sensing and Glc-signaling pathways are still largely unknown (Jang and Sheen, 1994).

Clearly, the apex was competent to respond to the floral stimulus, forming the sepal, stamen, and petal whorls soon after the completion of induction regardless of carbon supply. However, 1 d following induction, the *P. nil* apex may not have been competent to form carpels when cultured on 2% (w/v) Suc. In this instance we use an operational definition whereby competent cells or organs can respond in the expected manner in response to the appropriate developmental signal (Taiz and Zeiger, 1998). In other words, 1 d following induction, the apex may have been unable to become switched to carpel whorl morphogenesis. Alternatively, since the same apex would have formed carpels on Glc, perhaps this monosaccharide is required for carpel development. This would mean that on 2% (w/v) Suc, the apex may have carpel identity but be developmentally arrested. Either way, the data indicate remarkably different effects of Glc and Suc on development.

The longer interval of 5 to 6 d for carpel determination may be a period during which the meristem acquires competence to respond to the floral stimulus. This competence may be explicable entirely on morphological grounds. Note that the carpel whorl only starts to appear on d 6 following induction in vivo as the center of a considerably enlarged meristem begins to be consumed by the incipient gynoecium (Herbert et al., 1992) (Fig. 1). Therefore, 5 d are necessary for the apex to grow sufficiently for the appearance of carpel primordia, although increasing the level of Suc to 6% (w/v) shortened the carpel determination time to 3 d. As noted by McDaniel (1996), this suggests a requirement in the shoot apical meristem for a metabolic competence to facilitate floral morphogenesis, an argument that is germane to the carpels but not so obviously to the outer whorls of the *P. nil* flower.

In conclusion, Glc and Fru had some surprising effects on carpel whorl determination time, an effect that could not be reproduced by any of the carbohydrate-mannitol combinations except for Glc and mannitol (6% [w/v] Glc). The remarkable sensitivity of the shoot apical meristem of *P. nil* to Glc and Fru could help to explain the differences in patterns of determination reported for other species. For example, perhaps in *L. temulentum*, which exhibits coincident determination of all whorls (McDaniel et al., 1991), more monosaccharides are generated in the shoot apical meristem following induction compared with *S. coeli-rosa*, which exhibits sequential determination (Donnison and Francis, 1993). In *P. nil*, Glc and Fru treatments may result in rapid expression of homeotic genes that regulate gynoecium morphogenesis, thereby shortening the time required for determination of this whorl and allowing this mechanism to be studied at the molecular level.

In more general terms, our data are a reminder of the surprising effects carbohydrates can have on development. For example, callus from induced tobacco plants formed floral buds on a medium supplemented with Glc, but formed vegetative buds in the absence of Glc. However, callus from uninduced plants was unresponsive to Glc (Chailakhyan et al., 1975). In other words, Glc induced floral gene expression in florally competent cells. Still in question is the extent to which carbohydrates influence morphogenesis, either directly, through the induction of changes in gene expression, or indirectly, through alterations in metabolic state, which then permit particular modes of development.

MATERIALS AND METHODS

Growth Conditions

Seeds of *Pharbitis nil* Chois cv Violet (Muritane Seed Company, Kyoto) were selected for uniform size color and shape and scarified in 32 N H_2SO_4 at 27°C ± 1°C for 30 min (Herbert, 1991). The seeds were then washed twice in distilled water and immersed in running tap water at 25°C to 30°C for 2 h. Subsequently, they were abraded against a plastic mesh to further remove the surrounding testa, and then given an overnight imbibition of aerated distilled water (27°C ± 1°C employed throughout unless otherwise stated). Seeds that failed to germinate or produced radicles <15 mm in length were discarded, and the remainder sown in thoroughly moistened John Innes No.1 compost, with five plants per 75-mm plastic pot. These pots were then placed in gravel trays and watered from the base (approximately twice a week). The seedlings were induced to flower by growing them for 5 d under continuous light of 400 μmol m^{-2} s^{-1} provided by cool-white fluorescent tubes (Sylvania, London) at 27°C, followed by 48 h darkness, and then a return to continuous light (Durdan, 1998). At daily intervals following the inductive treatment, shoot tips were excised and shortened to approximately 10 mm of tissue and dissected to expose the shoot apical meristem and surrounding primordia. Each apex was then attached to a sample holder with a drop of Cryoembed (Bright Instrument Co., Cambridge, UK) and examined immediately with a low-vacuum scanning electron microscope (JSM–5200 LV, JEOL, London).

Through examination of floral apices at various times following induction in vivo, and using the method of Maeska and Ogawa (1994), we devised a simple numerical scheme to score the progressive stages of floral morphogenesis.

To ascertain when the floral stimulus started to move out of the cotyledons, these organs and any detectable leaf primordia were excised from each of 12 replicate plants at 0, 4, 8, 12, 16, 20, 24, and 48 h after the start of the inductive treatment. The plants were then returned to continuous light, and 4 weeks later scored for evidence of terminal flowers.

Tissue Culture

At various times (between 1 and 6 d) following the inductive treatment, shoot apices (the shoot apical meristem plus 4–5 leaf primordia plus 10 mm of basal tissue) were explanted on White's (1943) salt mixture solidified with 0.8% (w/v) agar (in vitro grade 1, Sigma, St. Louis) and supplemented with Suc (58, 116.8, or 175.3 mM), Glc, Fru (111, 222, or 333 mM for each), or equimolar 1:1 Glc:Fru (analar grade, British Drug House, Dorset, UK). From here on, we refer to these concentrations as 2% (w/v), 4% (w/v), or 6% (w/v). Basal medium was also supplemented with sugar mannitol combinations that would generate osmotic potentials equivalent to that of the 4% (w/v) and 6% (w/v) sugars (e.g. 20 g L^{-1} Suc + 10.38 g L^{-1} mannitol = −0.29 MPa ≡ ψs of 4% [w/v] Suc). In each case, the medium was balanced to a pH of 5.8.

In all experiments, cultures were maintained for 4 weeks at 23°C ± 1°C in non-inductive continuous light from cool-white fluorescent tubes giving a photon fluence rate of 10 to 25 μmol m^{-2} s^{-1}; higher fluence rates resulted in bleaching (Durdan, 1998). Four weeks after the start of culture, the shoot apices were assessed by dissecting them and noting the number of each type of floral organ using a microscope (SMZ, Nikon, Tokyo). At no time during this work did we ever observe primordia singly, nor did we observe floral reversion (for example, carpels were always observed as a fused ring).

In the 2% treatments, the experiment was repeated two to three times, and each time 10 apices were cultured per day per treatment. In the >2% treatments, more apices were cultured per day in single experiments: approximately 15 to 20 apices were cultured per day per treatment. In vitro data for d 4 and 5 are presented only for the 2% (w/v) Suc treatment, because at the time of these experiments we were unsure when the inner whorls would appear in vivo. Furthermore, sampling apices on d 4 and 5 became redundant because, in vivo, the stamens and petals appeared on d 5 and the carpels on d 6 (see Table I). In other words, determination times >4 d for the stamens and petals and >5 d for the carpels were not possible because by then these organs were already initiated in vivo.

Statistical Analyses

The data were analyzed using χ^2 ($P = 0.05$) or the non-parametric Mann-Whitney test ($P = 0.05$).

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