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A modular tray growth system for barley

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Running Head: barley growth system

Abstract

Determining when a barley plant starts and finishes meiosis is not trivial as when the spikelets undergo meiosis, the spike is not visible as it is still well within the leaf sheath on the developing tiller. This is a general constraint for any experiment involving meiosis, such as cytology, RNA extractions, or abiotic stress treatments aiming to target such a developmental stage. The lack of synchronicity between barley tillers within the same plant exacerbates the difficulty to determine the overall meiotic stage of a plant at a certain time.

Given the lack of a non-destructive staging system for predicting the entry into meiosis and the problems of working with large pot plant systems, a modular plant growing is proposed. This system enables the growth of a high number of plants in a small surface, each producing a single tiller. The modular tray system was used to generate a non-destructive prediction tool for meiosis by using external morphological features. As an example, the system is used here for heat treating F_1 plants in early meiosis stages to modify recombination.

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1. Introduction

The study of meiosis and recombination in plants requires a precise understanding of when and where a plant initiates floral development and undergoes meiosis. This is important because many experiments such as cytological, transcriptomics and protein studies, involve collecting tissue, usually anthers at certain meiotic stages from plants. In addition, other experiments may not require tissue collection, but still rely on estimating the meiotic stage of a plant for deciding when to apply treatments such as temperature stress to target certain stages of meiosis (Fig. 1), etc.

In barley, when the meiotic cells undergo differentiation, the spike is still inside the leaf sheath on the expanding tiller (barley stem), so it is not possible to assess the meiotic stage of the cells in a non-destructive way. Furthermore, the lack of synchronicity of meiosis between the different spikelets and tillers within the same plant, complicates the assessment of the meiotic stage of a plant.

The different cereal developmental scales available, such as the widely used Zadoks scale [1, 2] do not make a link between external developmental features and meiotic stage and do not make a link to spike growth at all until the ear starts to emerge. The Waddington scale [3] does measure the developmental growth of the sexual organs, but does not give any insight of meiotic progress at the cellular level, and requires the dissection and destruction of the material. Gómez and Wilson published a non-destructive method [4] that related spike size using an adaptation of the Zadoks scale to predict pollen stages and the anther development. Unfortunately, the earlier stages of meiotic progression were not differentiated. Another non-destructive system was published by Tracy et al., [5] using X-ray micro computed tomography (µCT) for floral stage phenotyping of *Arabidopsis thaliana* and barley cultivar Optic. This system provides a detailed internal 3D phenotype of flowers of living plants but is of limited use for larger scale application and did not differentiate meiotic stages. The meiotic entry and progress of barley is thus not covered by any published non-destructive staging system.

In this chapter, a new protocol for predicting meiotic stages that is more amenable to higher throughput is introduced. It uses modular trays widely used in some breeding applications, where plants are grown in small modules restricting their size and development, constraining them to develop a single tiller per plant, and to grow faster. Using staging data, a protocol has been devised that provides a non-destructive meiosis reference table for barley experiments and its use is demonstrated in an experiment that uses temperature treatments to modulate meiotic recombination.

2. Materials

2.1. Modular tray growing platform

The modular tray growing platform is prepared before sowing the plants.

1. Capillary matting: This matting is widely used in gardening and is made of a mixture of fabrics capable of holding and transporting large volumes of water. The watering matting is often sold in rolls and is cut when mounting the system. Example: AquaMat® from LBS.
2. Outer plastic tray: A large enough tray is required to contain the matting and the size of modular plant tray used inside. Plastic gardening trays, often known as propagator trays can be used. These trays require a system to allow the egress of water, so some holes are drilled in the corners of the tray if necessary (*see* **Note 1**, Fig. 2a) (e.g., G16B Garden tray, Garland Products Ltd).
3. Modular tray: These are plastic trays containing a large number of cells to grow plants. They are widely used in gardening, plant nurseries, and for some breeding applications such as speed breeding or single seed descent [6, 7]. The number and cell size can be variable, as an example (QPD104 trays, PG Horticulture) the following sizes can be found (*see* **Note 1**)
    - 104 round cell trays (13 × 8; approx. diameter: 3.5 cm/5 cm depth)
    - 96 square cell trays (12 × 8; approx. 3.7 × 3.7 cm/8 cm depth)
4. Containers for watering: Buckets, or plastic containers can be used to keep the water. The size must be suitable to fit in the space between the plastic tray and the bench.

5. Support for the trays (Fig. 3): The tray system needs to be supported to a height so that the watering buckets do fit underneath. As an example, inverted plant pots can be used for this task.
6. String/Twine as a support for the plants. Example: Gardening jute or polypropylene twine (Fig. 4).

2.2. Growing material

1. A controlled environment for growing the plants (*see* **Note 2**).
2. Soil (*see* **Note 3**).
3. Barley seeds. In the case of the present example cultivar "Morex" (six-rowed spring barley), cultivar "Bowman" (two-rowed spring barley), and F_1 seed produced from a cross between them are used.
4. Petri dishes. Variable size depending the number of seeds. In the present example 140 mm ø Petri dishes are used.
5. Sterile Distilled Water (SDW).
6. Filter paper to fit petri dishes (Whatman™). Variable size depending the number of seeds. In the present example 150 mm ø filter papers are used.
7. Parafilm™.

2.3. Staging material

1. Ruler.
2. Tweezers.
3. Optical microscope with graticule. Example: Olympus CK2® light microscope.
4. Microscope Slides. No coating required.
5. Coverslips (VWR).
6. Acetocarmine (as Chapter 15 of this book and [8]).

3. Methods

3.1. Modular tray platform preparation

1. Cut the capillary matting to the size and shape of the surface of the outer plastic tray (Fig. 2b, c).
2. Cut strips of capillary matting. They need to be long enough to cross the area of the plastic trays and hang below long enough to reach the depth of the water containers (Fig. 2b, c).
3. Place the matting strips on the tray. The strips can be positioned in different ways, such as a cross (Fig. 2b) or crossing the trays horizontally or vertically (*see* **Note 4**). The tips of the strips need to hang down the sides of the tray to where the watering system will be located.
4. Cover the strips by the square matting cut in step 1 (*see* **Note 5**).
5. Water the matting until is completely wet (*see* **Note 6**).
6. Fill the modular tray with the soil (*see* **Note 7**) and position it on the watering matting in the tray.
7. Mount the whole system on the supporting pots and place the water containers (filled with water, *see* **Note 8**) underneath with the matting strips in the water (Fig. 3, Fig. 4).

3.2. Sowing and plant growing

1. Place the seeds in petri dishes (around 30 seeds per plate) with filter paper and add SDW so that the paper is wet but does not drip if turned on the side. Prepare more seeds than needed, as not all of them may germinate (*see* **Note 9**). In the present example, 104 cell trays are used, and around 30% more seeds than needed are germinated.
2. Seal the petri dish with Parafilm™, and place the plate in the dark at RT.
3. Once the first root and coleoptile apices emerge from the seeds (Fig. 5b), sow them in the cells (coleoptile facing the top) (*see* **Note 10**), choosing healthy seeds with as little evident variation in terms of coleoptile emergence as possible.
4. Keep the capillary matting always wet. To achieve this in the initial stages, watering over the capillarity matting through the sides of the tray is required to ensure that the whole matting system including the strips is totally wet. Additional watering might be required on the matting before the root system is developed. Once the plants are 2-3 weeks old the watering should

work through capillary action from the watering containers, so only keeping them full of water is required.

5. Grow the plants (*see* **Note 11**) in the most controlled and constant conditions as possible. In the present example growth conditions in the cabinets is 18ºC day and 15ºC night for the control conditions before the treatment, and 30ºC day and 25ºC night for the heat shock conditions.

3.3. Collecting staging data

While the population grows, some plants are sampled, dissected, and staged for building the reference table that is used to predict meiosis. Ideally, a whole tray is used for this to ensure a reasonably sized dataset. It is important to sample only healthy plants, and discard all the plants that show problems, such as late or bad emergence, or sickness, like yellow leaves.

1. After the third week, dissect some plants at regular intervals to detect the first stages of spike morphogenesis (Waddington stages 1-2) [3].
2. Once the spike detected is around 3-4 mm, start sampling more frequently, by picking (ideally) three random plants every two or three days (*see* **Note 12**).
3. Measure the following morphological features (*see* **Note 13**):
    - Plant height (cm): Measure starting from the ground to the last unfolded leaf's ligule (*see* **Note 14**).
    - Number of unfolded leaves: Count the number of unfolded leaves in the main shoot (*see* **Note 15**).
    - Spike size (mm): Measure the total length of the spike.
    - Anther size in the middle of the spike (mm): Measure the length of the anther of a floret in the middle of the spike by using the graticule of the light microscope. It is taken from the middle of the spike as the most advanced meiotic stage in the spike is usually found here.

- Meiotic stage by acetocarmine spread (when possible): Place the same anther used to measure the length on a slide with a drop of Aceto-Carmine as described in Chapter 15 of this book and cover by a re-usable plastic coverslip. Push the coverslip gently with a rod glass to break the anther. Assess the meiotic stage under the light microscope using a magnification of 20× and 40× (Fig. 6). For this work four categories of meiotic stages are classified: Pre-meiosis ("Pre-meio"), Leptotene to Zygotene ("Lept.-Zyg."), Pachytene to Tetrad ("Pach.-Tet."), and Pollen (all stages after young pollen grains).

4. Once the three plants dissected show pollen, the sampling can be stopped, unless further stages are needed in the reference table.

3.4. Building a reference table

The measured variables, linked to the meiotic stage can be summarized in a table (like Table 1), which is used as reference to link non-destructive morphological features with the meiotic stage and the spike size or the anther size. This type of table can be produced by using the R script supplied, which uses the package "psych" (https://cran.r-project.org/web/packages/psych/psych.pdf) on the example dataset. In the example, samples from cultivars Bowman, Morex, and the F_1s from the cross between them are collected.

1. Open R Studio and import the file where the staging data is included (Example dataset provided).
2. Follow the instructions of the example R script included to generate the reference summary table. The outcome is a *csv* file table that can be then edited manually with a spreadsheet software to select only the desired information and tidy up the table for visualization (Table 1).

3.5. Using the reference table in temperature experiments

The following example shows how the non-destructive reference table for spike size and meiosis is used in a heat stress temperature experiment to study the effect of heat on meiotic recombination (*see* **Note 16**, Fig. 5):

1. Prepare a modular tray as in section 3.1 with barley seeds (in this case with F_1 seeds).
2. When the population of plants is getting close to the stage of treatment initiation (in number of leaves and height of plants), do whole tray staging but only for the plant height and number of unfolded leaves. In the present example, the temperature treatment aimed to cover the pre-meiotic and early meiotic stage, so the initiation of temperature stress was pre-meiosis.
3. When the mean of the tray for the number of leaves and height reaches the level associated with the desired meiotic stage to start the treatment, the batch is moved to the cabinet for heat treatment (30ºC day, 25ºC night). In this example this was done with a mean of 3.9 (±0.04) unfolded leaves and 15 cm (±0.43) height, based on the data presented in the reference table (*see* **Note 17**, Table 1).
4. As it was assumed the plants were in pre-meiosis, the control tray was moved to the Glasshouse at (20ºC day, 16ºC night).
5. The plants were kept in the heat treatment until it was considered the pre-meiotic stage and entry in meiosis was completed. In this case, this was when plants had a mean of 6.5 (±0.2) unfolded leaves and 25.6cm (±1.34) height (Table 1).
6. To assess recombination, F_2 seeds from both control and heat treatment are sown, and plants are genotyped. In this case using the BeadXpress® (Illumina) 96-SNP genotyping technology that uses a subset of the markers on the 9K Illumina SNP chip [9].
7. The comparison of the resultant genetic maps (calculated with MapDisto [10]) shows an increase of recombination in the heat-treated population compared to the control between the pericentromeric markers 11_10461 and 11_20969, from 9.5 cM under control conditions to 20.4 cM under heat stress (Fig. 7). These results indicate that the distribution of crossovers in barley can be modified by temperature stress, as it has been observed in previous studies [11].

4. Notes

1. The size of the outer tray or the modular cell tray does not need to be the same as in this protocol, smaller or bigger systems could be prepared by cutting or merging together modular trays.
2. The control of the environmental variables during the growth of the plants will depend on the place of the experiment and the resources available. Ideally plant growth cabinets present the best control of the environment, this being the crucial factor to ensure synchronicity between the plants and homogeneity between batches of trays.
3. The soil and substrate used will depend in the experiment. In the present example the soil used was general purpose mixture compost including: Peat, Sand, Limestone, Perlite, Celcote®, Osmocote®, and Exemptor®.
4. The position of the strips and the capillary matting need to ensure a flat surface in the tray, so that all the cells of the modular tray make contact with it.
5. The capillary matting system could be covered with a black micro-perforated plastic film (LBS) to maintain moisture better and avoid the formation of algae on the matting (Fig. 2d).
6. It is important to keep the matting wet all the times as if it gets dry the water transport can be compromised.
7. The soil in the cells of the modular trays must be filled equally. It can be compacted with the fingers to make sure all the cells are full of soil to the same level.
8. The watering system through capillarity could be used also to apply fertilizers, which could be mixed in the water container.
9. The seeds should be chosen with aim of ensuring as good quality of seeds and homogeneity between seeds as possible, in order to improve synchronicity in germination and growth.
10. To facilitate sowing, a hole can be formed in the soil (around 1 cm depth) of each of the cells using the back side of a thick pen, or the tip of a Falcon™ tube, for example.

11. The plants can be held by a support system to direct their growth, and to hold them erect and separated to avoid falling over each other (Fig. 4).
12. The aim of the sampling is to cover all the stages from the pre-meiotic stages to the end of meiosis. For this reason, the sampling frequency and intensity could be changed at early sampling to ensure that there are sufficient plants at later stages of particular interest.
13. Additional variables can be measured and included in the reference summary table to see if it can be improved or simplified. Some examples of more non-destructive variables include the number of days after sowing when the sample was collected, the Accumulated Growing Degree Days (AGDD) when the sample was collected, or the number of nodes detected in the stem.
14. The last height is taken to the flag-leaf´s ligule, even when the inflorescence has emerged completely, as the collar of the ear sometimes surpasses the flag-leaf, and sometimes does not.
15. As [2] describes, "A leaf can be described as unfolded when its ligule has emerged from the sheath of the preceding leaf". In this study, a decimal number was added after a comma, referring to the elongation of the next leaf to the one already unfolded from 1 to 10. This elongation was referred to the previous unfolded leaf; for example, if the 6^{th} leaf was the latest leaf emerging (still folded) and had reached a length of the last unfolded leaf (the 5^{th}), the stage would be 5.5.
16. The temperature experiment was carried out on F_1 plants of a cross between cultivars Morex and Bowman. The control conditions were in glasshouse with around 20ºC day and 16ºC night (Although ideally, it would be done also in a control growth cabinet), and the heat treatment was done in a growth cabinet (SANYO 1700) with 30ºC day and 25ºC night.
17. Most of the data in the reference summary table (Table 1) was generated with Morex and Bowman data, however the decisions were made on the F_1s. Whilst such fine scale genotypic differences should be taken into account if seed quantities allow it, it was assumed that such genetic variation was less than the within tray environmental variation shown by the staging data and thus ignored in this case.

Acknowledgments

The author would like to acknowledge Jim McNicol and BioSS for the advice and helpful discussions around the staging data and to Ed Byrne and KWS-UK for their advice and collaboration in the COMREC network. This research was funded by the European Community's Seventh Framework Programme FP7-PEOPLE-2013-ITN COMREC- 606956. I.C. was funded by the European Community's Seventh Framework Programme *FP7/2007-2013* under grant agreement n° 222883 MeioSys and by ERC advanced grant “Shuffle” (Project ID: 669182). L.R. and R.W. were funded from the Scottish Government’s Rural and Environment Science and Analytical Services Division Theme 2 Work Program 2.1.

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Figures

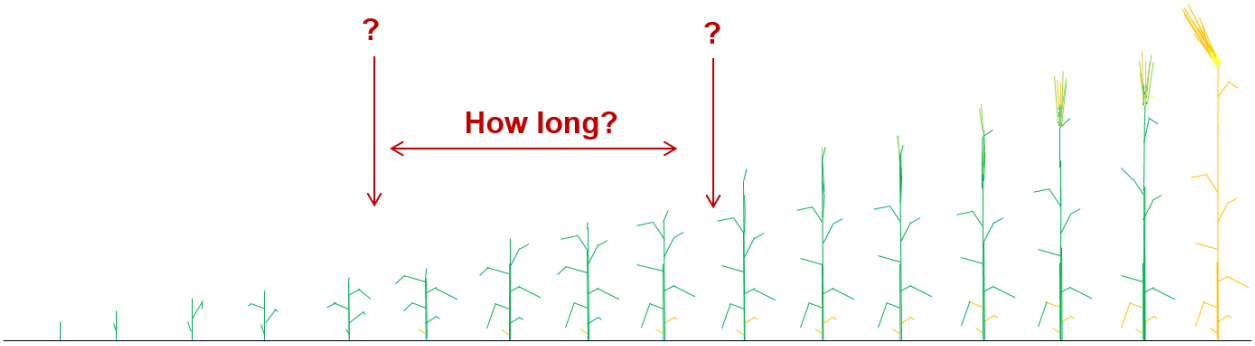

Fig. 1 A prediction of the internal floral development stage is required for example when applying a short temperature stress treatment targeting meiosis, in order to decide when to start and finish the treatment.

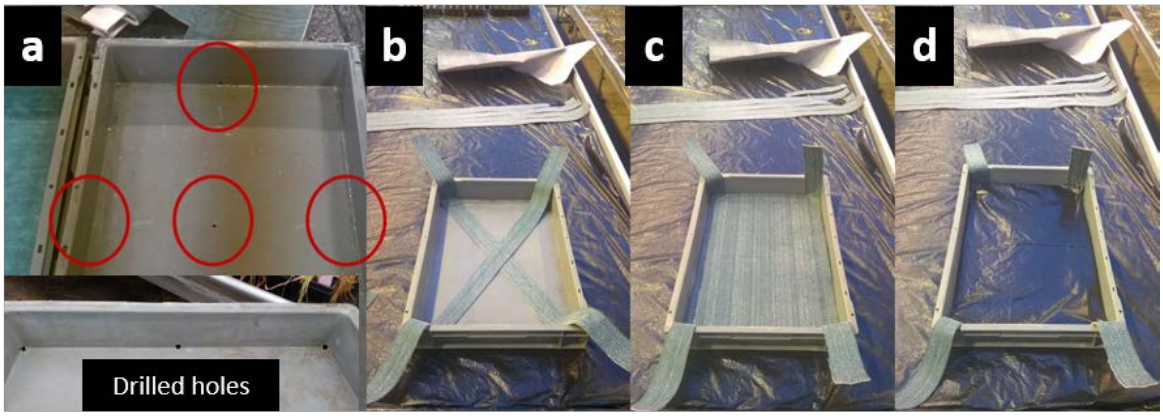

Fig. 2 Examples of different stages of mounting the watering system.

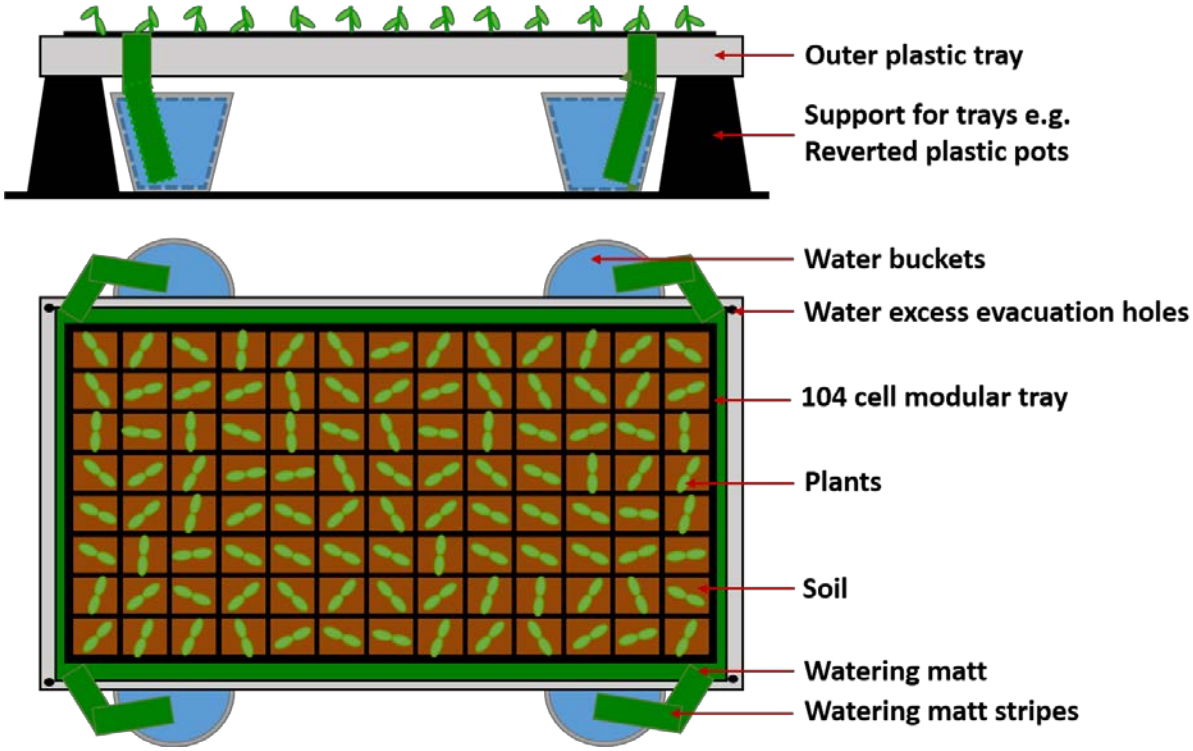

Fig. 3 Diagram of the modular tray system.

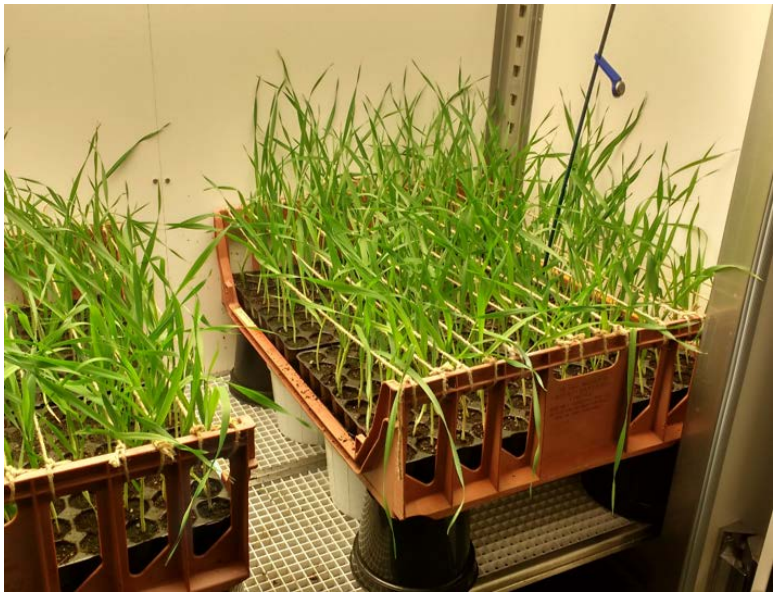

Fig. 4 Example of support system to hold the help the plants grow and avoid falling on each other.

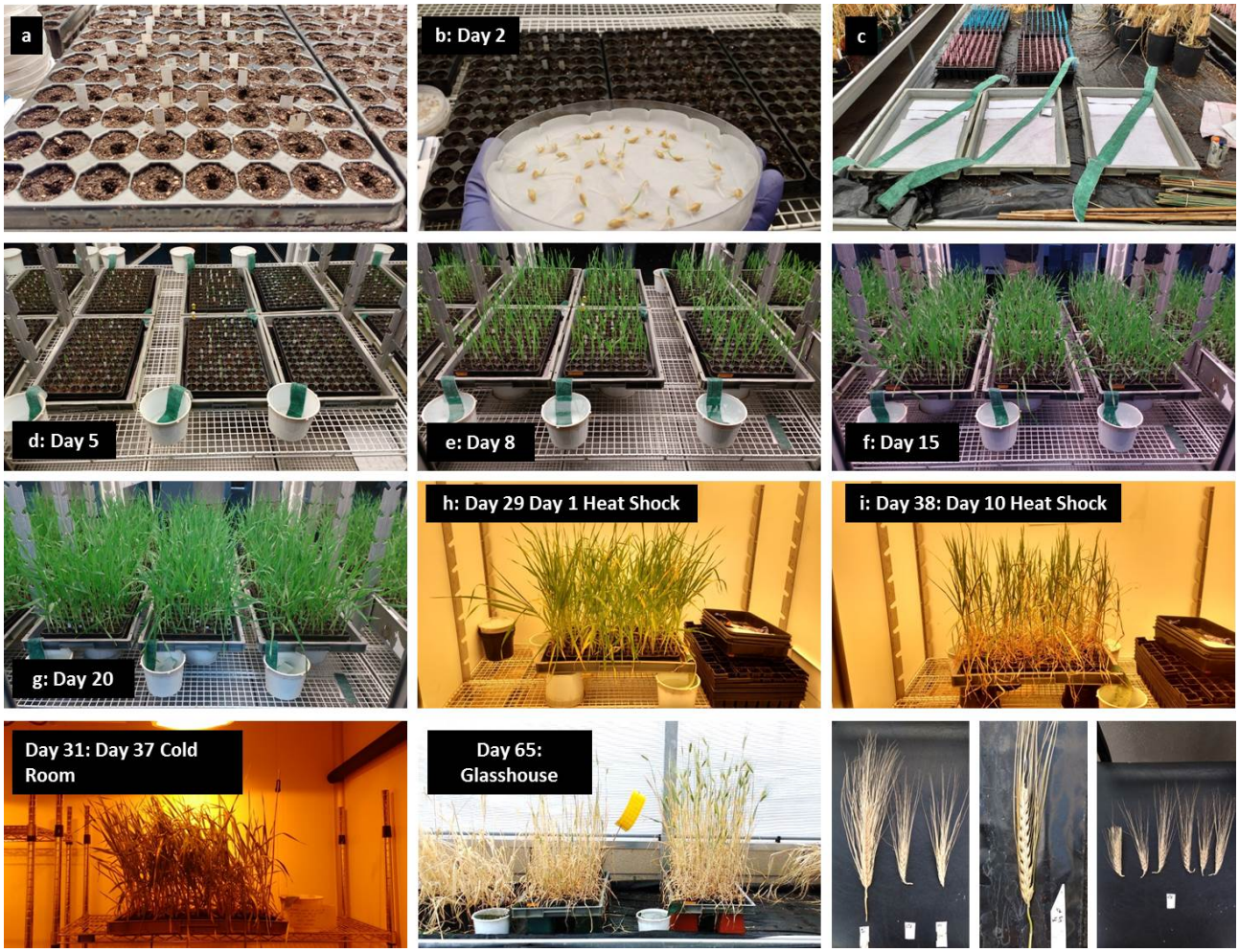

Fig. 5 Different stages of the modular tray system, temperature treatments, and harvesting.

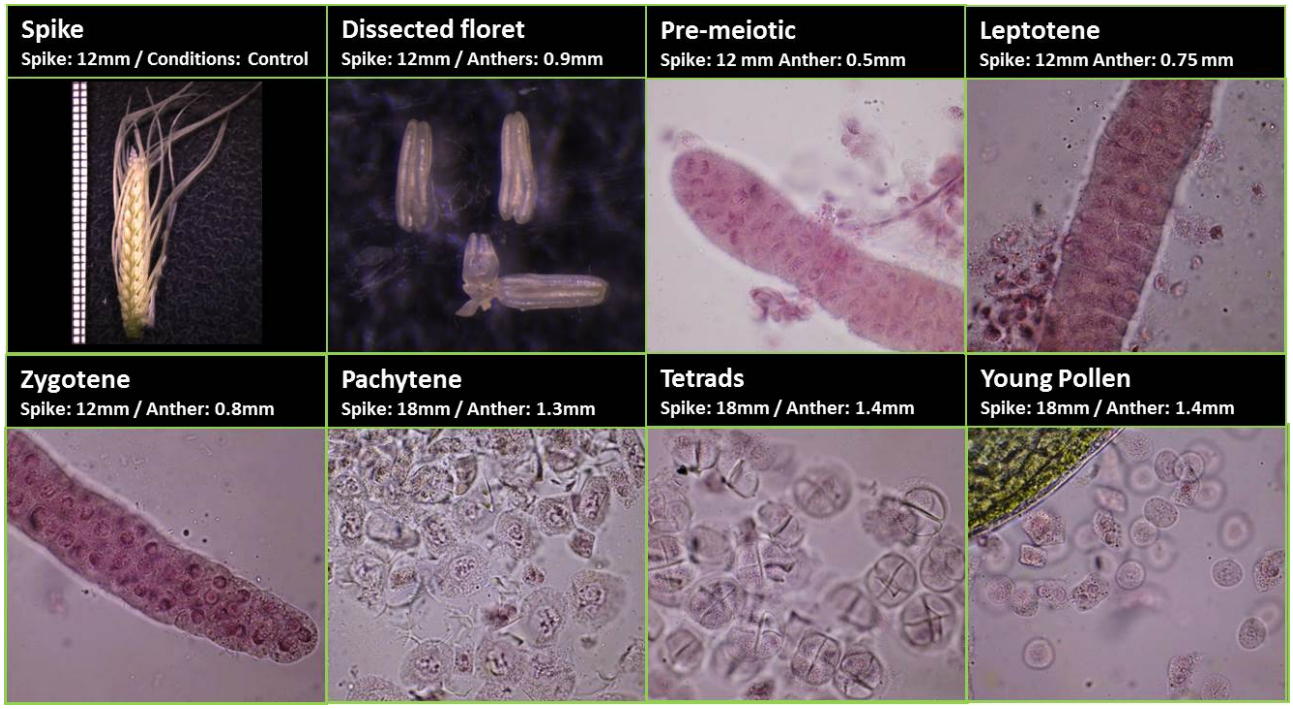

Fig. 6 Acetocarmine spreads and meiotic stages of barley anthers.

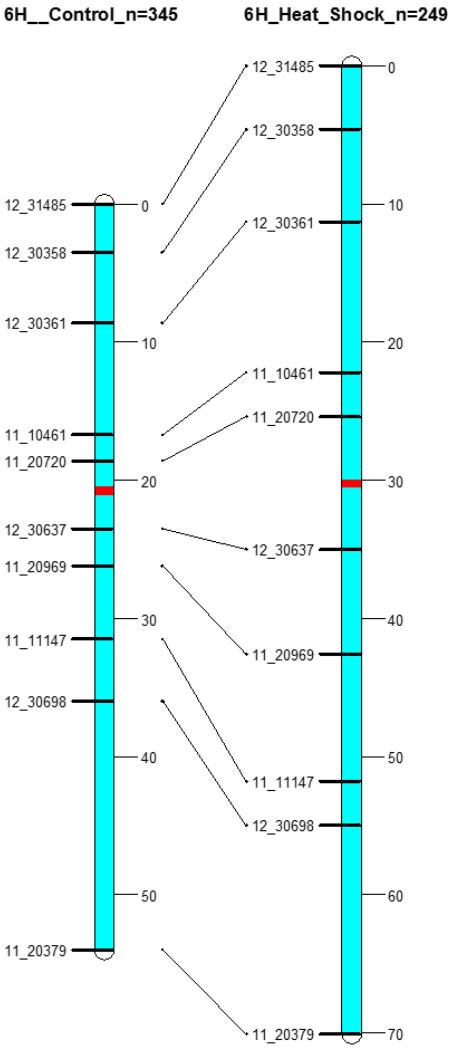

Fig. 7 Comparison of the genetic maps of chromosome 6H for control (left) and heat treated (right) conditions. The barley genomic zone based on [12] are coloured by Blue (as “Zone 2”). The red bar indicates the approximate position of the centromere.

Genotype	Meiotic Stage (Middle of the Spike)	Treat.	n	Mean Height (cm)	S.E.	Mean n. unfolded Leaves	S.E.	Mean Spike Size (mm)	S.E.	Mean Anther Size (mm)	S.E.
All	0_Pre-mei.	Ctr.	8	19.6	1.3	4.7	0.3	4	0.7	0.3	0.1
All	1_Lept.-Zyg.	Ctr.	7	26.9	1.6	5.6	0.3	9.3	0.8	0.7	0.1
All	2_Pach.-Tetr.	Ctr.	7	29.9	0.5	6.1	0.2	13.6	1.1	1	0.1
All	3_Pollen	Ctr.	7	36.1	2.1	7.6	0.5	27.1	2.9	1.6	0.2
All	0_Pre-mei.	H.S.	5	20.4	1.3	5.9	0.5	5.6	0.7	0.4	0
All	1_Lept.-Zyg.	H.S.	7	23.1	1.0	6.1	0.3	11.1	0.8	0.6	0
All	2_Pach.-Tetr.	H.S.	7	28.5	0.9	6.8	0.2	13.7	1.4	0.9	0.1
All	3_Pollen	H.S.	8	33.2	1.5	6.9	0.2	25.2	3.4	1.7	0.2

Table 1 Reference summary table with the means of different developmental features for each meiotic stage per treatment ("Treat."), (H.S.: Heat Shock, Ctr.: Control). All genotypes were used together to create this table.

R script

```
#################### REFERENCE TABLE FOR MEIOSIS PREDICTION ###################

# NEEDED PACKAGES
#readxl: to import the Excel dataset
library(readxl)
#Psych: as this package includes a function to calculated summary statistics
based on multiple variables
library("psych")

#Import the Excel file (can be done by hand by clicking on "Import Dataset")
#The name of the example data set is
“Modular_tray_grow_system_for_barley_Example_dataset"

#Save the data in "mydata"
mydata<-Modular_tray_grow_system_for_barley_Example_dataset

#Attach dataset to simplify the functions
attach(mydata)
```

```
#create a summary statistics table for the variables based on the Meiotic stage
("Meio"), and the Treatment ("Treat").

#Mean of Heights
describeBy(Height,list(Meio,Treat),mat=TRUE,digits=1,na.rm=TRUE)
#Mean of unfolded Leaves
describeBy(Leaves,list(Meio,Treat),mat=TRUE,digits=1,na.rm=TRUE)
#Mean of Spike sizes (mm)
describeBy(Spike,list(Meio,Treat),mat=TRUE,digits=1,na.rm=TRUE)
#Mean of Anther sizes (mm)
describeBy(Anther,list(Meio,Treat),mat=TRUE,digits=1,na.rm=TRUE)

#To create a general table including all the variables:

byHeight<-describeBy(Height,list(Meio,Treat),mat=TRUE,digits=1,na.rm=TRUE)
byHeight$variable<-"Plant height (cm)"
byLeaves<-describeBy(Leaves,list(Meio,Treat),mat=TRUE,digits=1,na.rm=TRUE)
byLeaves$variable<-"Number of unfolded leaves"
bySpike<-describeBy(Spike,list(Meio,Treat),mat=TRUE,digits=1,na.rm=TRUE)
bySpike$variable<-"Spike size (mm)"
byAnther<-describeBy(Anther,list(Meio,Treat),mat=TRUE,digits=1,na.rm=TRUE)
byAnther$variable<-"Anther size (mm)"
#Create total table
summary_reference_table <- rbind(byHeight,byLeaves,bySpike,byAnther)
#save table in Coma Separated Values File
write.table(summary_reference_table, "Summary_Refefernce_Table.csv")


#Detach dataset
detach(mydata)

#Open csv. file and change the format to visualize the desired parts of the
data.

################################################################################
```