Journal of Plant Physiology 214 (2017) 7–15

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Research paper

FLC expression is down-regulated by cold treatment in *Diplotaxis tenuifolia* (wild rocket), but flowering time is unaffected

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ARTICLE INFO

Keywords:
Bolting time
Diplotaxis tenuifolia
Photoperiod
Vernalization
Wild rocket

ABSTRACT

Wild rocket (*Diplotaxis tenuifolia*) has become a very popular salad leaf due to its peppery taste. It is part of the *Brassicaceae* family and thus has a high level of homology at the DNA level to other Brassica species including *Arabidopsis thaliana*. The vernalization and photoperiodic requirements of wild rocket have not been reported to date. Photoperiodic experiments described here demonstrate that rocket is a facultative long day plant. To investigate the vernalization requirement, both seed and young plants were given vernalization treatments at 4 °C for different lengths of time. A rocket homologue of *FLOWERING LOCUS C* (*DtFLC*) was isolated and shown to functionally complement the *Arabidopsis* FRI^{+}*flc3* null mutant. Whilst the expression of *DtFLC* was significantly reduced after just one week of cold treatment, cold treatments of two to eight weeks had no significant effect on bolting time of wild rocket indicating that rocket does not have a vernalization requirement. These findings illustrate that important fundamental differences can exist between model and crop plant species, such as in this case where down-regulation of *DtFLC* expression does not enable earlier flowering in wild rocket as it does in *Arabidopsis* and many other Brassica species.

1. Introduction

Wild rocket (*Diplotaxis tenuifolia*) has increased in popularity over the last 20 years in the leafy salads market (Chun et al., 2013), in the UK alone over 80 tons of rocket is consumed per week (Gill, 2008). The genus *Diplotaxis* is found in the *Brassicaceae* family in the *Oleracea* clade (Arias and Pires, 2012) and is therefore closely related to species *Brassica rapa*, *Brassica juncea*, *Brassica napus* and *Brassica oleracea,* as well as *Arabidopsis thaliana* (Arabidopsis). Flowering is undesirable in commercial rocket production as pre-harvest flowering can lead to the crop being unsaleable (Fig. 1a), despite this very little work has been reported on the control of flowering in wild rocket. Here we investigate the vernalization and photoperiodic requirements of this relatively new salad crop species.

Regulation of flowering time involves a complex gene network which allows a plant to respond to both internal and external signals in order to control the timing of the transition from the vegetative phase to the reproductive phase (Srikanth and Schmid, 2011). The timing of this transition is crucial to enable reproduction to occur when the plant is at its fittest and the environmental conditions are most favorable (Thomas et al., 2006). Most research into flowering time has been conducted in the model plant Arabidopsis through the generation and testing of many mutant and transgenic overexpression lines to elucidate gene function (Corbesier and Coupland, 2005). Using the knowledge gained from Arabidopsis and applying it to crop species is helping our understanding of the control of flowering in crop plants. Currently there are six key pathways that regulate flowering time: the photoperiodic, autonomous, vernalization, gibberellic acid, age dependent and ambient temperature pathways (Brambilla and Fornara, 2013; Fornara et al., 2010; Jarillo and Pineiro, 2011; Srikanth and Schmid, 2011). All of these pathways converge on the floral pathway integrator genes such as *FLOWERING LOCUS T* and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* which activate the downstream floral meristem identity genes. When flowering is induced the shoot apical meristem changes from forming vegetative tissues such as leaves to form flowers.

Vernalization is the attainment or acceleration of floral competence as a consequence of prolonged exposure to cold temperature (Chouard, 1960). In vernalization-responsive plant species, vernalization conveys the ability of the plant to flower and can greatly reduce the time it takes to flower (Guo et al., 2004; Hackett and Hartmann, 1967; Jung and Mueller, 2009; Strange et al., 2011). The cold treatment causes epigenetic changes that reduce levels of a floral repressor gene

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http://dx.doi.org/10.1016/j.jplph.2017.03.015
Received 7 February 2017; Received in revised form 22 March 2017; Accepted 24 March 2017
Available online 28 March 2017

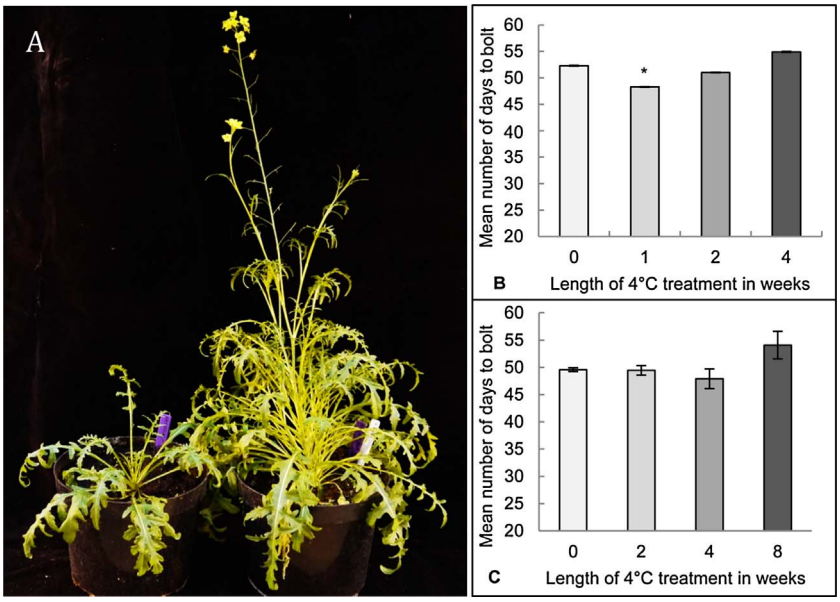

Fig. 1. Effect of vernalization at 4 °C on D.tenuifolia.A) Photo of a non-bolting rocket plant (left) and one that has bolted and flowered (right).B) The mean number of days to bolt of plants where the seed was subjected to cold treatment at 4 °C for one, two or four weeks. Bars show mean ± SE (n = 14 (0 weeks), n = 15 (1 week and 2 weeks), n = 10 (4 weeks)). Student's *t*-test was used to compare the number of days to bolt of each vernalization treatment (one, two and four weeks) against the ambient conditions (0 weeks). *Statistical significance of $p < 0.05$C) Mean number of days to bolt for 4 week old plants subjected to cold treatment at 4 °C for two, four and eight weeks. The length of the vernalization treatment was subtracted from the number of days to bolt. Bars show mean ± SE (n = 14 (0 weeks), n = 15 (2 weeks and 4 weeks), n = 13 (8 weeks)). Student's *t*-test was used to compare the number of days to bolt of each vernalization treatment (2, 4 and 8 weeks) against the ambient conditions (0 weeks). There was no significant difference in number of days to bolt between vernalization treatments and ambient conditions ($p < 0.05$)

FLOWERING LOCUS C (*FLC*). When the plant is returned to a higher temperature these epigenetic changes are stable enough to maintain the repression of *FLC* expression and the reduced levels of this repressor thus enables flowering to be induced (Boss et al., 2004). Studies of Arabidopsis ecotypes show that there are differences in the requirement for vernalization. Rapid cycling ecotypes will flower without the need for a vernalization treatment, whereas winter annual ecotypes will flower very late unless exposed to a period of vernalization (Nordborg and Bergelson, 1999; Wang, 2014). It was found that these vernalization response phenotypes all relate to the levels of expression of *FLC* (Song et al., 2012). Plants which have a requirement for vernalization tend to be those from seasonal climates where adaptation to extended periods of cold can be advantageous. The stage in the plant's lifecycle which is responsive to vernalization varies between species as does the length of treatment needed in order to fully satisfy the vernalization requirement. Arabidopsis is able to respond to vernalization as both seed and young plants (Nordborg and Bergelson, 1999; Strange et al., 2011), whereas many *Brassica* species only respond to a vernalization treatment as young plants (Jung and Mueller, 2009). Rocket is closely related to both Arabidopsis and *Brassica* and it is not currently known at which stage of its lifecycle it is able to respond, what the optimum vernalization period might be, or even if it has a vernalization response at all.

The ability of a plant to measure and respond to the length of day, or photoperiod, is often critical to its growth and development, particularly in the timing of the floral transition. Most plants fall into one of three main photoperiodic categories; long day (LD) plants which flower when the photoperiod is longer than a critical length, short day (SD) plants which flower when the photoperiod is shorter than a critical length, and day neutral plants which flower regardless of the photoperiod (Matsoukas et al., 2012). LD plants flower quite quickly in LD, but if subjected to SD conditions they will flower much more slowly (facultative response) or not at all (obligate response), the opposite being true for SD plants.

Understanding the vernalization and photoperiod requirements of commercial crops, and the underlying genes that control these responses in these crops, is of huge benefit to crop breeding programs aiming to create varieties that can be grown successfully in a range of latitudes and climatic conditions. In this paper, we investigate the photoperiodic and vernalization responses of *D.tenuifolia*. We show that wild rocket is a facultative long day plant and that it does not respond to vernalization treatments of several weeks duration even though the expression of the rocket *FLC* orthologue is strongly reduced by these treatments.

2. Materials and methods

2.1. Plant material

Elsoms Seeds Ltd provided seed of *Diplotaxis tenuifolia* bred through four generations of single seed descent for uniformity of leaf shape, color, and bolting time. *Arabidopsis thaliana* mutant FRI^{+} *flc3* null (Col0 background) seed was originally sourced from NASC (http://arabidopsis.info/) and used in the complementation experiments.

2.2. DNA and RNA extraction

Rocket genomic DNA was extracted using CTAB extraction method adapted from Stewart and Via (1993) on frozen leaf material, which was ground to a powder using a Dremel Drill with a 1.5 ml Eppendorf tube drill bit. 300 µl CTAB B buffer (100 mM Tris/Cl pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% hexadecyltrimethyl ammoniumbromide) was added and homogenized. Samples were incubated at 65 °C for 30 min and centrifuged. The supernatant was removed into a new tube and twice extracted using 300 µl Chloroform:Isoamyl alcohol (24:1) and centrifugation. The aqueous top phase was transferred each time. 300 µl CTAB C buffer (1% hexadecyltrimethyl ammoniumbromide, 10 mM EDTA, 50 mM Tris/Cl pH 8.0) was added and left overnight at room temperature. On day two, the tubes were centrifuged and the pellet dissolved in 400 µl 1 M CsCl. DNA is then precipitated using 100% Ethanol and centrifuged. The pellet was then washed twice using 70% Ethanol before drying and resuspending in 25 µl TE buffer (pH 8) with RNase A (Invitrogen) (20 µg/ml).

RNA extraction was done using the Z6 extraction buffer (containing