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Temperature-dependent shade avoidance involves the receptor-like kinase ERECTA

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SUMMARY

Plants detect the presence of neighbouring vegetation by monitoring changes in the ratio of red (R) to far-red (FR) wavelengths (R:FR) in ambient light. Reductions in R:FR are perceived by the phytochrome family of plant photoreceptors and initiate a suite of developmental responses termed the shade avoidance syndrome. These include increased elongation growth of stems and petioles, enabling plants to overtop competing vegetation. The majority of shade avoidance experiments are performed at standard laboratory growing temperatures (>20°C). In these conditions, elongation responses to low R:FR are often accompanied by reductions in leaf development and accumulation of plant biomass. Here we investigated shade avoidance responses at a cooler temperature (16°C). In these conditions, *Arabidopsis thaliana* displays considerable low R:FR-mediated increases in leaf area, with reduced low R:FR-mediated petiole elongation and leaf hyponasty responses. In Landsberg *erecta*, these strikingly different shade avoidance phenotypes are accompanied by increased leaf thickness, increased biomass and an altered metabolite profile. At 16°C, low R:FR treatment results in the accumulation of soluble sugars and metabolites associated with cold acclimation. Analyses of natural genetic variation in shade avoidance responses at 16°C have revealed a regulatory role for the receptor-like kinase ERECTA.

Keywords: shade avoidance, temperature, *Arabidopsis thaliana*, phytochrome, R:FR, ERECTA.

INTRODUCTION

Selective absorption of red and blue wavelengths by photosynthetic tissue results in a reduction in the red to far-red ratio (R:FR) of light transmitted through and reflected from living vegetation. In many higher plants, perception of low R:FR by the phytochrome photoreceptors results in a suite of developmental responses termed the 'shade avoidance syndrome' (SAS). These include elongation of stems and petioles, leaf hyponasty, increased apical dominance and accelerated flowering, responses which often occur at the expense of leaf development and plant biomass (Smith and Whitelam, 1997; Franklin, 2008). The perception of reflected FR signals within dense stands enables plants to detect the proximity of competitors and initiate light-foraging behaviour before canopy closure (Ballaré *et al.*, 1987, 1990). An additional role for green light signals in this process has recently been suggested (Zheng *et al.*, 2011) When shaded, plants are subject to reduced quantities of photosynthetically active radiation and increased concentrations of the gaseous hormone ethylene (Ballaré *et al.*, 1987, 1991; Finlayson *et al.*, 1999; Pierik *et al.*, 2003, 2004; Vandenbussche *et al.*, 2003; Keuskamp *et al.*, 2012). The integration of these signals enables plants to accurately determine the impending threat of light limitation and initiate an appropriate developmental response.

The molecular mechanisms controlling shade avoidance have started to emerge following the identification of genes responsive to low R:FR and simulated shade treatments in Arabidopsis (Devlin *et al.*, 2003; Salter *et al.*, 2003; Sessa *et al.*, 2005). Elongation growth is driven by low R:FR-mediated increases in auxin synthesis (Tao *et al.*, 2008) and cell wall modifying protein expression (Sasidharan *et al.*, 2010). Three basic helix–loop–helix (bHLH) transcriptional regulators of the PHYTOCHROME INTERACTING FACTOR (PIF) subfamily, PIF4, PIF5 and PIF7, perform a fundamental role in this process (Nozue *et al.*, 2007;

Lorrain *et al.*, 2008; Li *et al.*, 2012) with other family members performing overlapping functions (Leivar *et al.*, 2012). In low R:FR conditions, PIF4 and PIF5 stability is enhanced whilst the PIF-binding DELLA family of growth suppressors are degraded (Djakovic-Petrovic *et al.*, 2007; Lorrain *et al.*, 2008; de Lucas *et al.*, 2008). Elongation growth is limited by the formation of non-DNA-binding heterodimers between PIF4/PIF5 and atypical bHLH proteins such as LONG HYPOCOTYL IN FAR RED (HFR1) (Sessa *et al.*, 2005; Hornitschek *et al.*, 2009) and PHYTOCHROME RAPIDLY-REGULATED 1/2 (PAR1/PAR2) (Galstyan *et al.*, 2011).

Accumulating evidence suggests that phytochrome and temperature signalling processes are tightly integrated. Studies of the light-mediated suppression of flowering have shown that phytochrome hierarchy is regulated by temperature. In warmer environments, phytochrome B (phyB) dominates this response, with phyE performing a more significant role under cooler conditions (Halliday and Whitelam, 2003; Halliday *et al.*, 2003). The integration of phytochrome and temperature signals has also been observed in other processes, including the regulation of seed germination (Penfield *et al.*, 2005; Heschel *et al.*, 2007; Donohue *et al.*, 2008), leaf hyponasty (van Zanten *et al.*, 2009b), plant cold acclimation (Franklin and Whitelam, 2007; Catalá *et al.*, 2011; Lee and Thomashow, 2012) and maintenance of plant biomass (Foreman *et al.*, 2011). Recently, PIF4 has been shown to regulate both elongation growth and flowering at high temperature, thereby acting as a node of crosstalk between light and temperature signalling pathways (Koini *et al.*, 2009; Stavang *et al.*, 2009; Foreman *et al.*, 2011; Franklin *et al.*, 2011; Kumar *et al.*, 2012).

The majority of shade avoidance studies use standard Arabidopsis protocols, with growth temperatures in excess of 20°C (Fankhauser and Casal, 2004). However, at least two investigations have suggested that temperature may affect these responses. Growth of *Abutilon theophrasti* in 26°C/20°C cycles enhanced stem elongation responses to low R:FR when compared with plants grown in 18°C/16°C cycles (Weinig, 2000), while Arabidopsis plants grown at 16°C displayed increased leaf area responses to low R:FR (Franklin *et al.*, 2003). The latter is in stark contrast to the characterised SAS (Smith and Whitelam, 1997) and may suggest the existence of temperature-dependent regulatory mechanisms. To further understand how temperature controls shade avoidance in Arabidopsis, we quantitatively analysed morphological and metabolomic changes to low R:FR at different temperatures. Natural genetic variation in selected responses was then used to identify regulatory components. At cooler temperature, Landsberg *erecta* (L*er*) plants dramatically increased leaf blade cell expansion in low R:FR. The production of larger, thicker, leaves was accompanied by increased levels of compatible solutes. The characteristic SAS phenotypes, petiole elongation and leaf hyponasty, were significantly reduced when compared with responses at 22°C. In contrast to L*er* and the other accessions tested, the Cape Verde Islands (Cvi) accession of Arabidopsis displayed similar shade avoidance responses at cool and warm temperatures. Analysis of natural genetic variation in temperature-dependent shade avoidance has revealed a key role for the receptor-like kinase, ERECTA.

RESULTS

Temperature regulates leaf expansion responses to low R:FR

At 22°C, Arabidopsis plants (L*er*) grown in low R:FR displayed elongated petioles and reduced leaf thickness (Figures 1a,b,d,e and 2a, Table 1), consistent with the well-established SAS (Smith and Whitelam, 1997). No statistically significant differences in leaf area were observed between high and low R:FR-grown plants in our conditions (Figure 2b). These data are supported by scanning electron microscopy (SEM) studies showing no significant difference in leaf pavement cell area between high and low R:FR-grown plants at 22°C (Figure 1c,f, Table 1). When grown at a cooler temperature (16°C), however, a strikingly different shade avoidance phenotype was observed. Leaves developed in low R:FR at 16°C displayed no significant petiole elongation, but dramatically increased leaf area and thickness when compared with high R:FR-grown controls (Franklin *et al.*, 2003; Figures 1g–l and 2b, Table 1). Microscopy of leaf cross-sections showed low R:FR-grown leaves to display enhanced cell expansion of all cell types and an increased volume of air space within the mesophyll tissue (Figure 1h,k). The latter is characteristic of leaves undergoing cell expansion (Pyke *et al.*, 1991). Consistent with leaf area measurements, SEM analysis revealed a significant increase in leaf pavement cell area in low R:FR plants when compared with high R:FR-grown controls (Figure 1 i,l, Table 1). Together, these data suggest that the regulation of leaf growth by temperature and light quality is mediated primarily by changes in cell expansion. Similar responses were observed in continuous and light/dark cycles of low R:FR (Figure 1 and Figure S1 in Supporting Information). In agreement with previously published observations (Halliday and Whitelam, 2003), flowering was accelerated in low R:FR at 16°C and 22°C (Figure S2), suggesting that temperature predominantly regulates architectural responses to low R:FR.

Temperature regulates low R:FR-mediated changes in biomass and metabolite content

The increased leaf area observed in low R:FR-grown L*er* plants at 16°C was accompanied by a striking increase in whole plant biomass. This is in stark contrast to experiments at 22°C, where low R:FR-grown plants displayed a

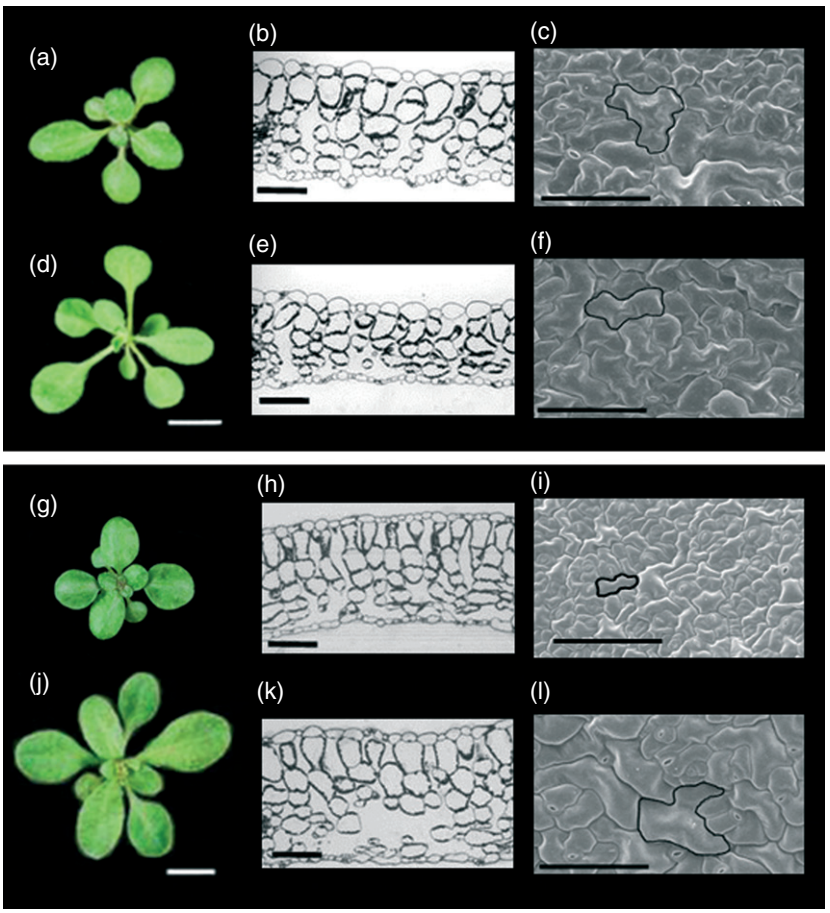

Figure 1. Shade avoidance responses at 16°C and 22°C.
Phenotypes of Arabidopsis plants (L*er*) grown in continuous high (a) and low (d) red:far-red (R:FR) ratio light at 22°C and continuous high (g) and low (j) R:FR ratio light at 16°C. Scale bars represent 10 mm. Light microscopy images of leaf cross sections (b,e,h,k) and SEM images of leaf pavement cells (c,f,i,l) are included from fully expanded rosette leaves. Scale bars represent 100 μm. Tissue sections were excised for microscopy when plants grown in low R:FR showed a 10-mm bolt.

Table 1 Leaf thickness and pavement cell area of the largest rosette leaf of plants grown in high and low red:far-red (R:FR) at 16°C and 22°C. Values are shown ± SE

	Leaf thickness (μm)	Pavement cell area (μm^2)
High R:FR 16°C	236.1 ± 7.0	806.5 ± 45.6
Low R:FR 16°C	294.2 ± 11.9*	2695.3 ± 124.5*
High R:FR 22°C	207.6 ± 5.4	2094.9 ± 204.1
Low R:FR 22°C	177.7 ± 12.3*	1733.9 ± 93.1

*Significant when compared to high R:FR-grown control ($P < 0.05$).

lower plant biomass than high R:FR-grown controls (Figure 2c). At cooler temperature, low R:FR-mediated increases in biomass were restricted to shoots, with no significant increases observed in roots (high R:FR = 0.9 ± 0.02 mg; low R:FR = 0.89 ± 0.09 mg). The shade avoidance syndrome is also characterized by a reduction in chlorophyll synthesis and an altered chlorophyll *a:b* ratio (Smith and Whitelam, 1997). In this study, low R:FR-grown L*er* leaves displayed a reduced chlorophyll content and increased chlorophyll *a:b* ratio at both temperatures (Figure 3a,b). To further investigate differences in leaf development between shade avoidance at 16°C and 22°C in these plants, we analysed soluble sugars and metabolite contents. Leaves grown in low R:FR at 16°C displayed a significantly higher concentration of soluble sugars than high R:FR-grown controls. In contrast, no significant difference was observed between high and low R:FR-grown plants at 22°C (Figure 3c). These data are supported by gas chromatography–mass spectrometry (GC-MS) metabolite profiles which showed low R:FR-grown plants at 16°C (but not 22°C) to display increased sucrose content (1.55 ×; $P = 0.013$; Table S1). We analysed GC-MS data for other major metabolites displaying differential regulation by low R:FR at 16°C and 22°C. The metabolite displaying the most striking temperature-dependent low R:FR-mediated change in levels was glycine. Plants grown in low R:FR at 16°C displayed a significant increase in glycine (3.07 ×; $P = 0.002$), when compared with high R:FR-grown controls. At 22°C, a relatively low glycine content was observed in both high and low R:FR (Figure 3d, Table S1). Other metabolites that increased in low R:FR at 16°C (but not 22°C) included citric acid (1.64 ×; $P = 0.022$), 5-oxo-proline (1.69 ×; $P = 0.030$) and glutamic acid (2.14 ×; $P = 0.069$). Several major metabolites were significantly reduced by low R:FR at 22°C, but not 16°C (Table S1), including phytol (0.40 ×; $P = 0.035$), β-sitosterol (0.62 ×; $P = 0.008$), fucosterol (0.62 ×; $P = 0.015$),

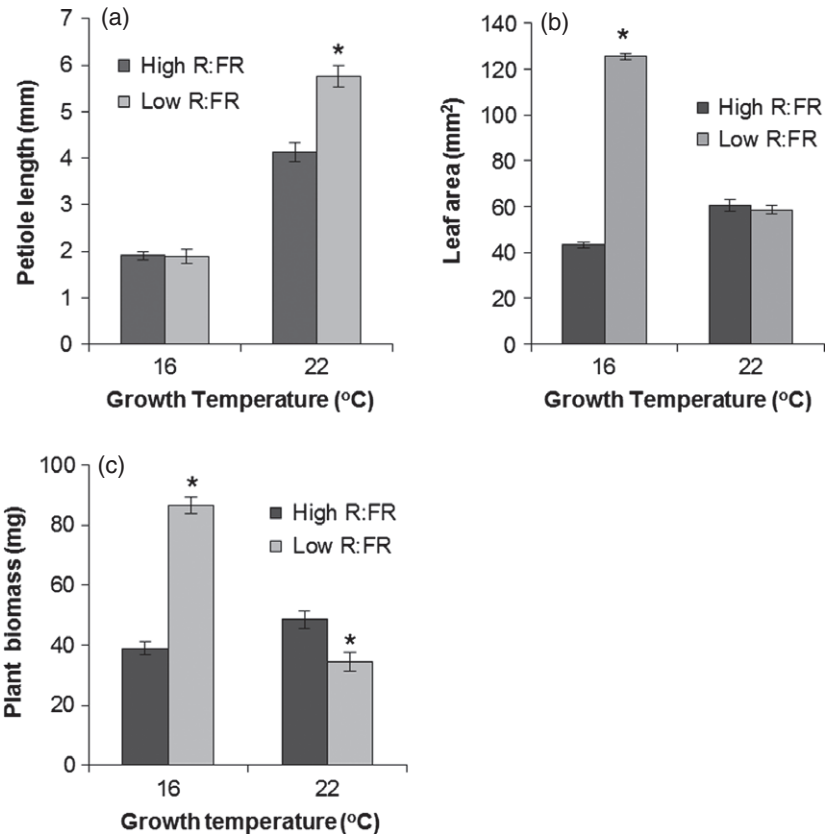

Figure 2. Temperature regulates low red:far-red (R:FR)-induced changes in leaf architecture and plant biomass.
Petiole length (a), leaf area (b) and plant biomass (c) of plants grown in high and low R:FR at 16°C and 22°C. Measurements were taken when low R:FR-grown plants displayed a 10-mm bolt ($n \geq 8$). Bars represent SE. *Significant when compared with high R:FR control using a Student's *t*-test ($P < 0.05$).

campesterol (0.66 ×; $P = 0.007$), linoleic acid (0.66 ×; $P = 0.015$), glycerol (0.67 ×; $P = 0.004$) and palmitic acid (0.84 ×; $P = 0.009$). These data show that in L*er,* information on both temperature and light quality is integrated to regulate plant metabolite content and could suggest the existence of different, temperature-dependent shade avoidance responses.

Natural genetic variation exists in shade avoidance responses at cool temperatures

In contrast to L*er*, Cvi plants displayed a similar shade avoidance responses at both 16°C and 22°C, showing significantly elongated petioles and hyponastic leaves, with only a small increase in leaf area at the lower temperature (Figure 4a–d). In high R:FR, leaf angles were lower at 16°C than at 22°C in both accessions, but still increased in response to low R:FR. Consistent with Van Zanten *et al.* (2009a) and van Zanten *et al.* (2010a,b), the Cvi accession displayed higher leaf angles than L*er* in all environmental conditions (Figure 4b). To investigate the molecular basis of this natural genetic variation, a L*er* × Cvi near isogenic line (NIL) population (Keurentjes *et al.*, 2007) was grown in high and low R:FR at 16°C. NIL 2-8 (containing a 2.3 Mb region of Cvi on chromosome 2) displayed a petiole elongation response similar to Cvi (Figure 4c) and a smaller leaf expansion response than its L*er* parent (Figure 4d). Comparative analysis of NILs overlapping the Cvi introgression narrowed the quantitative trait locus (QTL) to the 1.3 Mb region, which was further reduced to a approximately 0.45 Mb region between markers 11166049 and 11614873 by fine mapping (Table S2; Clark *et al.*, 2007). This region contains *ERECTA*, a gene previously established to perform a role in plant architecture and leaf development (Swarup *et al.*, 1999; Shpak *et al.*, 2003; van Zanten *et al.*, 2009b; Tisné *et al.*, 2011). *ERECTA* was therefore selected as a candidate gene for further analysis.

As a first step, the localization of *ERECTA* expression in low R:FR was investigated through analysis of *pER::*GUS fusions in both L*er* and Cvi plants grown in high and low R:FR at 16°C and 22°C (Figure 5a). A promoter region of 636 bp containing all the essential *cis*-regulatory elements for correct spatial and temporal expression was selected (Yokoyama *et al.*, 1998). Comparative analysis of *ERECTA* promoter sequences revealed three polymorphisms between L*er* and Cvi (Table S3). Accession-specific promoters were therefore used in each construct. In L*er* rosettes, GUS expression was predominantly localized to the apical meristem and elongating petioles (Figure 5a). In Cvi rosettes, GUS expression displayed strong expression in elongating petioles, but was also present in leaf blades in all conditions (Figure 5a). Full *ERECTA* transcription

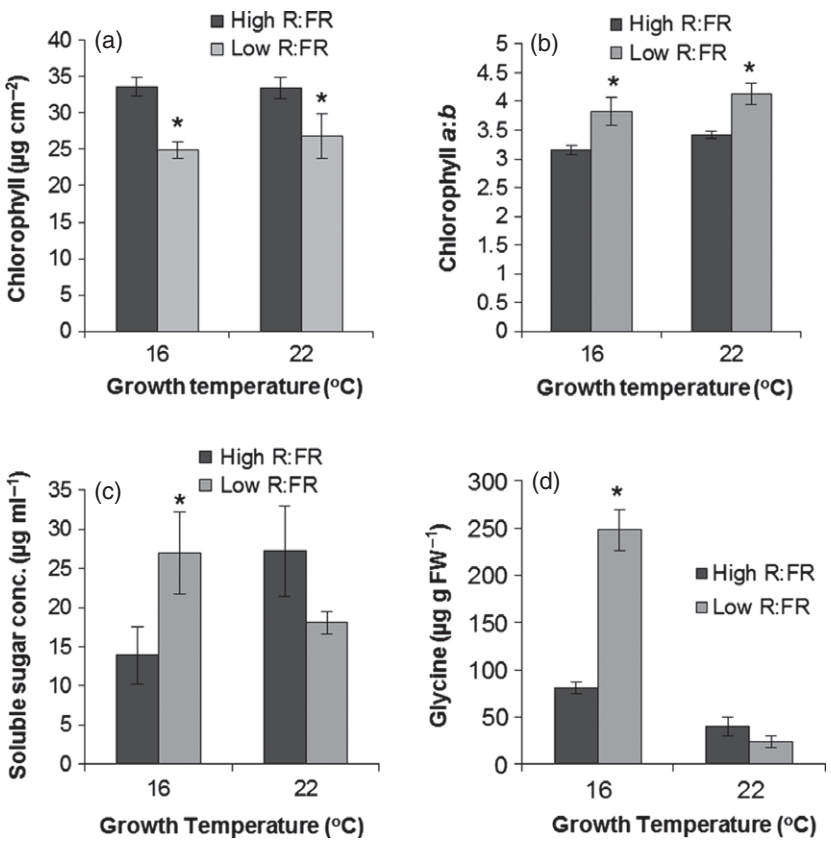

Figure 3. Temperature regulates low red:far-red (R:FR)-induced changes in metabolite accumulation.
Leaf chlorophyll content (a), leaf chlorophyll *a*:*b* ratio (b), soluble sugar content (c) and glycine content (d) of plants grown in high and low R:FR at 16°C and 22°C. Chlorophyll ($n = 5$) and metabolites ($n = 3$) were analysed 2 days prior to the bolting of low R:FR-grown plants. Bars represent SE. *Significant when compared with high R:FR control using a Student's *t*-test ($P < 0.05$).

requires introns (Karve *et al.*, 2012). We therefore measured endogenous transcript levels to quantitatively investigate the effects of low R:FR treatment on *ERECTA* expression. Both Cvi and La-0 accessions were analysed following a short (24 h) and prolonged (given until 2 days prior to bolting; 2 dpb) low R:FR treatment (La-0 is the parental line to L*er*, expressing functional *ERECTA*). No significant low R:FR-mediated increases in *ERECTA* transcript abundance were observed in La-0 or Cvi at either temperature, as analysed by a Student's *t*-test (Figure 5b). Significant decreases in *ERECTA* transcript abundance were observed in Cvi plants treated with low R:FR for 24 h at 16°C ($P = 0.026$) and 2 dpb at 22°C ($P = 0.045$). Similar, but less significant, reductions in *ERECTA* transcript abundance were observed following prolonged low R:FR treatment (2 dpb) in 22°C-grown La-0 ($P = 0.06$) and 16°C-grown Cvi plants ($P = 0.059$; Figure 5b). Differences in *ERECTA* localisation between L*er* and Cvi were also observed in seedlings. In L*er*, GUS expression was predominantly localized to the tip of the elongating hypocotyl, meristem and cotyledon petioles. In Cvi, GUS expression was recorded throughout the cotyledons, cotyledon petioles and hypocotyls (Figure S3). Together, localization data support a role for ERECTA in regulating elongation growth. Differences in *ERECTA* localization and abundance between Landsberg and Cvi accessions may reflect polymorphisms in their promoter sequences (Table S3).

ERECTA regulates petiole elongation in shade avoidance

The involvement of ERECTA in regulating low R:FR-mediated leaf development was investigated through analyses of mutants and complemented lines in a variety of accessions. The ERECTA-deficient accession L*er* was compared with its parent line Landsberg (La-0) and a complemented L*er*::ER line. Columbia (Col) wild-type plants were compared with mutants *er-1* and *er-105.* In addition, the ERECTA-deficient accessions Vancouver (Van-0) and Hiroshima (Hir-1) were compared with the complemented lines Van-0::ER and Hir-1::ER, respectively (van Zanten *et al.*, 2010a,b).

At 16°C, the presence of functional ERECTA restored low R:FR-mediated petiole elongation to the Landsberg background (Figures 6a,b and S4). At 22°C, the presence of functional ERECTA enhanced petiole elongation in both high and low R:FR (Figures 6a,c and S4). In the Columbia background, loss of functional ERECTA reduced petiole elongation responses to low R:FR at 16°C and 22°C, a result which was more pronounced in the *er-1* mutant (Figure 6a–c). A small petiole elongation response to low R:FR was observed in Van-0 plants at 16°C. This was considerably enhanced by complementation with *ERECTA* (Figures 6b

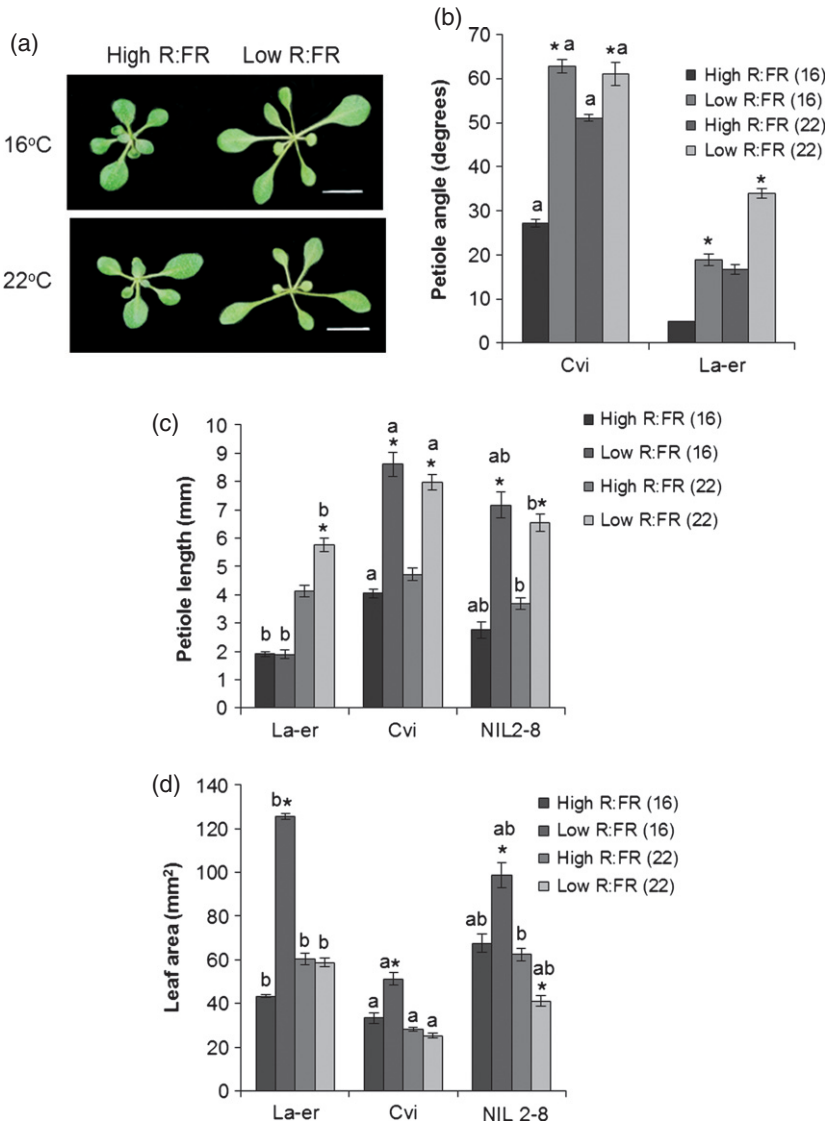

Figure 4. Cvi displays similar shade avoidance behaviour at 16°C and 22°C. (a) Cvi grown in high and low red:far-red (R:FR) at 16°C and 22°C. (b) Leaf angles in L*er* (La-er) and Cvi following 5 days in low R:FR. (c) Petiole lengths and (d) leaf area measurements of the largest rosette leaf in L*er*, Cvi and NIL2-8 grown in high and low R:FR at 16°C and 22°C. Photographs and measurements of petiole length and leaf area were taken when plants in low R:FR displayed a 10-mm bolt ($n = 16$). Bars represent SE. *Significant difference when compared to high R:FR-grown control using a Student's t-test ($P < 0.05$). [a]Significant difference when compared with L*er* in the same conditions ($P < 0.05$). [b]Significant difference when compared with Cvi in the same conditions ($P < 0.05$).

and S4). No petiole elongation response to low R:FR was observed in Hir-1 plants at 16°C, but was restored by complementation with ERECTA (Figures 6b and S4). At 22°C, ERECTA complementation enhanced low R:FR-mediated petiole elongation in both Van-0 and Hir-1 backgrounds (Figures 6c and S4). In summary, ERECTA promotes low R:FR-mediated petiole elongation. This is effect is more striking at cool temperatures, where petiole elongation responses to low R:FR are reduced compared with 22°C-grown plants (Col, Van-0) or absent (L*er*, Hir-1).

The presence of functional ERECTA modified leaf blade expansion in an accession-dependent manner at both temperatures (Figure 6d,e). No clear role could be identified for ERECTA in regulating leaf area or hypocotyl elongation responses to low R:FR (Figures 6a,d,e and S5), confirming a largely petiole-specific function in shade avoidance. All *ERECTA*-expressing and -deficient lines responded to low R:FR by significantly increasing leaf angles at both 16°C and 22°C (Figure S6). Growth in high R:FR at 16°C resulted in very low leaf angles in all lines. In the Landsberg background, loss of ERECTA significantly reduced leaf angle in other conditions. An opposite, but minor, effect was observed in the Col background, suggesting the influence of ERECTA on leaf angle to be background specific (Figure S6).

DISCUSSION

Studies of plant shade avoidance using standard laboratory growing protocols have resulted in establishment of the SAS, a suite of developmental responses initiated following perception of reduced R:FR (Smith and Whitelam, 1997). Rapid elongation of stems and petioles is therefore often considered to be the most significant architectural adaptation displayed by adult plants to the threat of light

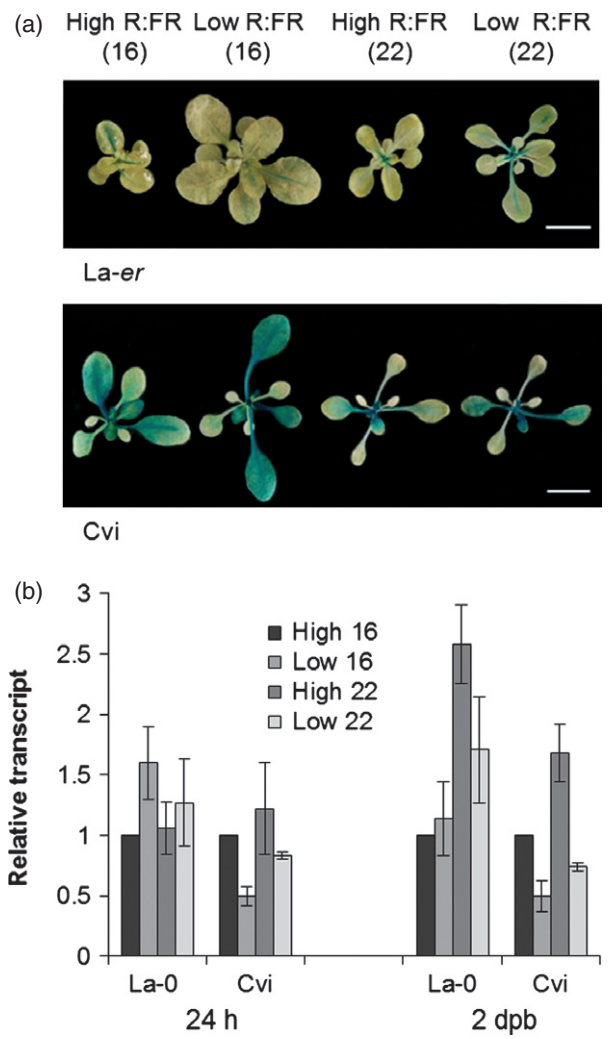

Figure 5. *ERECTA* expression is localised in elongating petioles in L*er* and Cvi backgrounds.
(a) *pERECTA*::GUS expression was analysed in L*er* (La-*er*) and Cvi plants grown in high and low red:far-red (R:FR) at 16°C and 22°C at 2 days prior to bolting (2 dpb). Scale bar represents 10 mm. (b) Transcript abundance of *ERECTA* in La-0 and Cvi plants following 24 h and prolonged (2 dpb) low R:FR treatment.

limitation (Franklin, 2008). Reductions in leaf development and plant biomass are generally regarded as an unavoidable consequence or necessary prerequisite of the reallocation of resources to support axis elongation and precocious flowering (Kasperbauer *et al.*, 1984; Keiller and Smith, 1989; Robson *et al.*, 1993; Devlin *et al.*, 1996; Tsukaya *et al.*, 2002). Here, we have shown that plant architectural responses to low R:FR are determined by the integration of light and temperature signals and can, in cooler environments, involve significant increases in leaf area and plant biomass. The acceleration of flowering by low R:FR was temperature-independent (Figure S2), providing further support for the existence of separate signalling mechanisms regulating light quality-mediated changes in plant architecture and floral induction (Botto and Smith, 2002; Cerdán and Chory, 2003).

Reductions in leaf chlorophyll content and altered chlorophyll *a* to chlorophyll *b* ratios (chl *a*:*b*) are characteristic of the SAS (Smith and Whitelam, 1997). Chlorophyll *b* is predominantly associated with the light-harvesting antenna complexes of photosystem II and reduced chl *a*:*b* ratios have been reported to correlate with increased blue light harvesting capacity (Yamazaki *et al.*, 2005). Similar reductions in both chlorophyll content and chl *a*:*b* ratio were recorded in low R:FR-grown L*er* plants at 16°C and 22°C (Figure 3a,b) suggesting pigment composition to be regulated independently of plant architecture.

An expanding body of evidence suggests that elongation responses observed in the SAS involve auxin biosynthesis and are regulated by the PIF and DELLA families of transcriptional regulators through targeted expression of downstream genes (reviewed in Franklin, 2008). In contrast, little is known about low R:FR-mediated signalling at cooler temperatures. Microarray analysis has shown that plants treated with low R:FR at 16°C but not 22°C display increased expression of *COLD REGULATED* (*COR*) genes (Franklin and Whitelam, 2007). These are downstream components of the C-repeat Binding Factor (CBF) regulon, expressed during cold acclimation (Thomashow, 2010). Expression of the CBF pathway results in a variety of metabolic changes which enhance the tolerance of plants to subzero temperatures (Cook *et al.*, 2004). We observed L*er* plants grown in low R:FR at 16°C to display enhanced levels of soluble sugars, especially sucrose, nitrogenous metabolites, including glycine, 5-oxo-proline and glutamic acid, and citric acid (Figure 3c,d, Table S1), consistent with expression of the CBF regulon in these conditions (Cook *et al.*, 2004; Franklin and Whitelam, 2007). In contrast, plants grown in low R:FR at 22°C displayed reductions in major lipid metabolites, including plant sterols, fatty acids and glycerol that was not evident at 16°C (Table S1).

The subtropical Cvi accession of Arabidopsis has been shown to display lower freezing tolerance than temperate accessions such as Wassilewskija and L*er*, resulting, in part, from a deletion in the *CBF2* promoter (Cook *et al.*, 2004; Alonso-Blanco *et al.*, 2005). We observed Cvi plants to display phenotypes similar to the SAS at both 16°C and 22°C, suggesting an impairment in cool temperature shade avoidance responses (Figure 4a). Furthermore, in high R:FR at 16°C, the Cvi accession displayed petiole elongation and leaf hyponasty responses similar to L*er* and Col plants grown at 22°C, suggesting a constitutive 'warm temperature' phenotype (Figures 4, 6, S4 and S6; Crawford *et al.*, 2012). Variation in shade avoidance responses at cool temperature between L*er* × Cvi NIL lines led us to investigate the role of ERECTA (Figure 4c,d). The *ERECTA* gene encodes a leucine-rich repeat (LRR) receptor-like kinase (Torii *et al.*, 1996) with a broad range of functions in plant

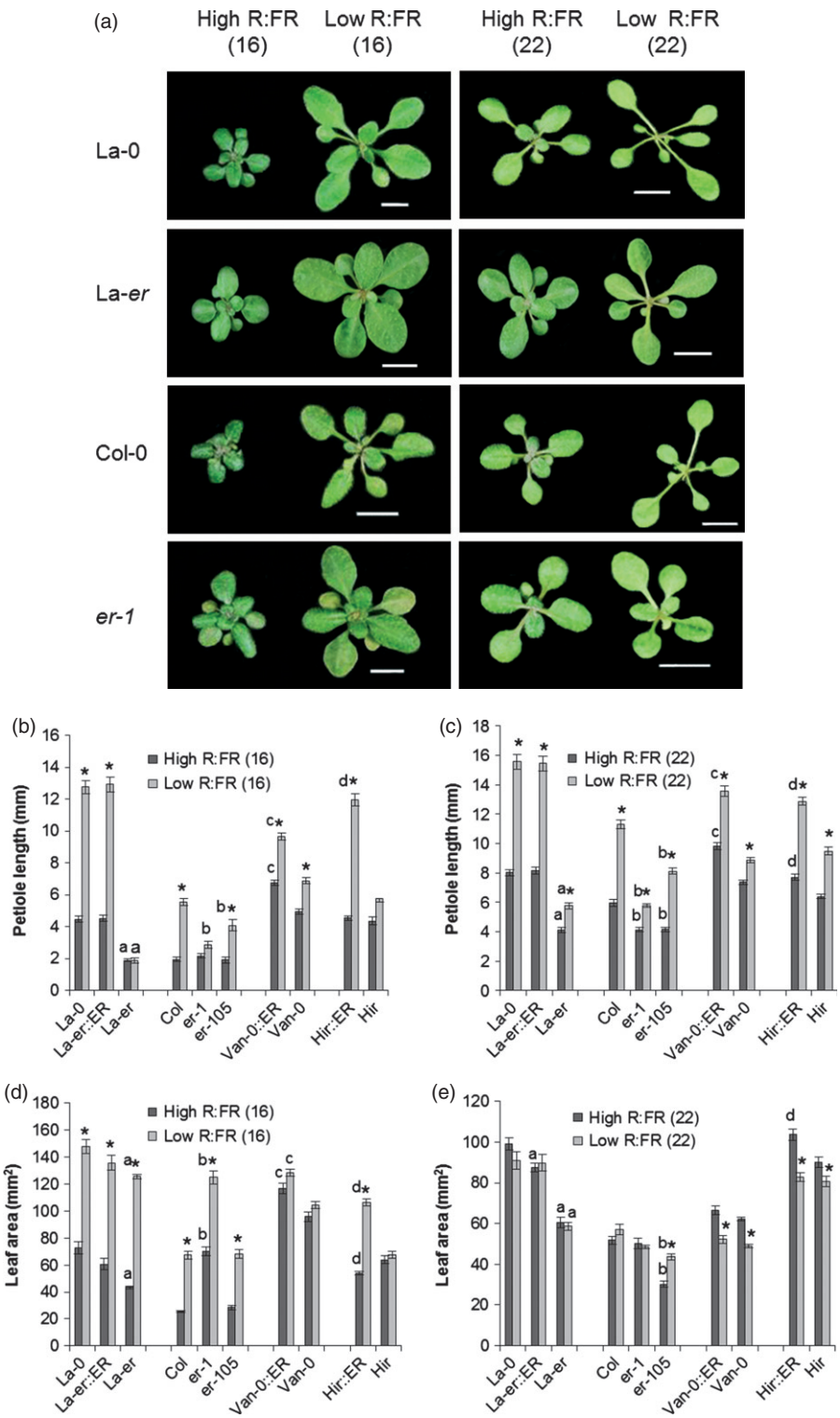

Figure 6. ERECTA regulates petiole elongation in shade avoidance.
(a) ERECTA-deficient and -expressing plants grown in high and low red:far-red (R:FR) at 16°C and 22°C. Scale bar represents 10 mm. Petiole lengths (b,c) and areas (d,e) of the largest rosette leaf from wild type (WT) and ERECTA-deficient plants grown as in (a). Van-0 and Hir are ERECTA-deficient accessions, shown with complemented lines. Measurements were performed when plants in low R:FR displayed a 10-mm bolt ($n \geq 8$). Bars represent SE. *Significant when compared with high R:FR control using a Student's *t*-test ($P < 0.05$). [a]Significant difference when compared with La-0 in the same conditions ($P < 0.05$). [b]Significant difference when compared with Col in the same conditions ($P < 0.05$). [c]Significant difference when compared with Van-0 in the same conditions ($P < 0.05$). [d]Significant difference when compared with Hir in the same conditions ($P < 0.05$). La-er = Landsberg *erecta*.

development, including organ size, pathogen resistance and transpiration efficiency (reviewed in van Zanten *et al.*, 2009b). The QTL analysis of a L*er* × Cvi recombinant inbred line (RIL) population has previously suggested that the *erecta* mutation could be linked to a short petiole phenotype (Swarup *et al.,* 1999). More recently, ERECTA has been shown to regulate leaf hyponasty in response to a low quantity of light and cell expansion in leaf blades, suggesting a key regulatory role in leaf development (van Zanten *et al.*, 2010a,b; Tisné *et al.*, 2011). Our data suggest a major role for ERECTA in regulating petiole elongation during shade avoidance, particularly at cool temperatures (Figures 6 and S4). The Columbia *er-1* mutant phenotypically mimicked L*er* and displayed a more extreme impairment of low R:FR-mediated petiole elongation than the null *er-105* allele (Figure 6). L*er* plants carry a mutation in the kinase domain of ERECTA (Torii *et al.*, 1996). Expression of a truncated *ERECTA* fragment lacking this kinase domain has been shown to confer dominant-negative interference with endogenous ERECTA function and enhance growth defects in a null mutant background (Shpak *et al.*, 2003). The authors suggest that expression of mutated ERECTA may deplete receptor ligands and/or interact with shared receptors, thereby interfering with other signalling pathways. In this way, expression of mutated *ERECTA* may confer exaggerated mutant phenotypes when compared with null lines. In accordance with studies of leaf hyponasty (van Zanten *et al.*, 2010a,b), the effectiveness of ERECTA in promoting petiole elongation was strongly dependent on genetic background. At 16°C, low R:FR-mediated petiole elongation was greatest in La-0 and Hir backgrounds, with smaller effects recorded in Col and Van-0 (Figures 6, S4). Expression of *pER::*GUS was strongest in elongating stems and apical meristems, supporting a role in elongation growth (Figures 5a and S3; Yokoyama *et al.*, 1998). In both seedlings and adult rosettes, greater staining was observed in Cvi plants, consistent with their elongated architecture (Figures 5a and S3). The response of *ERECTA* transcript levels to low R:FR was, again, background- and temperature-dependent. No effect of low R:FR was observed in 22°C-grown La-0 plants following a 24-h treatment. These data are consistent with published microarrays from Columbia plants showing no significant increase in *ERECTA* expression following 24 h of simulated shade treatment at 21°C (Sessa *et al.*, 2005). Decreases in *ERECTA* transcript abundance were generally observed in Cvi plants following low R:FR treatment at both temperatures (Figure 5b). In La-0, this decrease was only observed following prolonged low R:FR treatment at 22°C. Low R:FR-mediated down-regulation of *ERECTA* transcript abundance may act to limit petiole elongation in an accession- and temperature-dependent manner.

Further understanding of the role of ERECTA in promoting petiole elongation requires the identification of downstream targets. Transcriptional profiling approaches in seedlings have revealed regulatory networks in the ERECTA signal transduction cascade, including a suite of WRKY transcription factors (Terpstra *et al.*, 2010). Transcriptional profiling of the SAS has revealed a number of key marker genes, displaying increased transcript abundance in low R:FR. These include the transcription factors *PIF3-LIKE 1* (*PIL1-*; Salter *et al.*, 2003) and *ATHB2* (Steindler *et al.*, 1999). Both genes displayed increased transcript abundance following prolonged low R:FR treatment of adult plants at 16°C, with a similar, but reduced response observed at 22°C (Figure S7). No clear role could be identified for ERECTA in regulating these responses (Figure S7).

Together, our data show that the responses of Arabidopsis to low R:FR are conditioned by temperature in an accession-dependent manner. In warm environments, low R:FR initiates the SAS and plants forage for light via leaf elevation (Figure 1; Smith and Whitelam, 1997). The magnitude of petiole elongation growth is controlled, in part, by the action of ERECTA. The SAS phenotypically resembles growth at high temperature (Gray *et al.*, 1998; Balasubramanian *et al.*, 2006; Koini *et al.*, 2009; Kumar *et al.*, 2012). These responses share molecular signalling mechanisms, including PIF4-mediated auxin biosynthesis (Franklin *et al.*, 2011; Hornitschek *et al.* 2012; Li *et al.*, 2012). Complementary action of auxin and ERECTA signalling has been observed in inflorescence elongation (Woodward *et al.*, 2005; Uchida *et al.*, 2012) and may contribute to the control of petiole elongation in shade avoidance. The low R:FR-responsive gene, *ATHB2*, has been shown to regulate auxin sensitivity (Kunihiro *et al.*, 2011), but does not appear to be regulated by ERECTA.

It has recently been suggested that the elongated architecture observed in plants grown at high temperature enhances leaf cooling capacity through increasing leaf separation and height from the soil surface (Crawford *et al.*, 2012). Interestingly, ERECTA has also been shown to affect transpiration efficiency, through regulation of stomatal density and leaf ultrastructure (Masle *et al.*, 2005).The SAS may therefore be a favourable light-foraging strategy in hot, well-watered environments.

Growth at cooler temperatures results in a more compact plant stature, reduced leaf area, increased leaf thickness and delayed flowering (Blázquez *et al.*, 2003; Halliday *et al.*, 2003; Atkin *et al.*, 2006). With the exception of Cvi, petiole elongation and leaf hyponasty responses to low R:FR were reduced at cooler temperatures in all accessions tested (Figures 1, 2a, 4b,c, 6 and S5). Leaf blades, however, displayed dramatic low R:FR-mediated expansion (Figure 6d,e). Such responses may represent an effective light-foraging strategy in cooler environments. The expanded leaves of L*er* plants grown in low R:FR at 16°C additionally displayed elevated levels of soluble sugars and cold

acclimation products (Figures 1–3 and Table 1; Franklin and Whitelam, 2007). Unsurprisingly, these plants have been shown to display enhanced tolerance to freezing temperatures (Franklin and Whitelam, 2007). The regulation of plant shade avoidance by ambient temperature may therefore serve to maximise light-foraging potential in a manner which minimises future injury from heat or freezing stresses.

EXPERIMENTAL PROCEDURES

Plant material

La-0 (N1298), L*er* (NW20), L*er*::ER (N163), Cvi-1 (N8580), Col-1 (N3176), *er-1* (N3378) and *er-105* (N89504) lines were obtained from the Nottingham Arabidopsis Stock Centre (http://arabidopsis.info/). Hir-1, Hir::ER, Van-0 and Van-0::ER lines were provided by Martijn van Zanten (Utrecht University, The Netherlands). The L*er* × Cvi NIL population (Keurentjes *et al.*, 2007) was provided by Malcolm Bennett (University of Nottingham, UK). Experiments were performed with the Arabidopsis accession L*er* unless otherwise stated.

Plant growth

Seeds were sown directly onto Lehle medium (Lehle Seeds, http://www.arabidopsis.com/) supplemented with 0.8% (w/v) agar. After 4 days' stratification in darkness at 4°C, seedlings were germinated under 8-h light/16-h dark cycles at 19°C. Following 7 days of growth, uniformly sized individuals were transplanted to a 3:1 mixture of compost:horticultural silver sand. After an additional 7 days of growth under the same conditions, plants were transferred at the four-leaf stage to experimental light regimes at 22°C and 16°C. Low R:FR ratio treatments were initiated following 24-h acclimation. For all experiments, plants were grown in controlled growth chambers (Fi-troton 600H, Sanyo Gallenkamp, http://www.sanyo-biomedical.co.uk/ and Microclima 1600E, Snijder Scientific, http://www.snijders-scientific.nl/). White light was provided by cool-white fluorescent tubes (400–700 nm) at a continuous photon irradiance of 130 μmol m^{-2} sec^{-1} unless otherwise stated. Low R:FR experiments were performed with supplementary arrays of FR LEDs positioned overhead (λ_{max} 735 nm). For these experiments, plants received the same photon irradiance of photosynthetically active radiation, but with a R:FR of 0.1. All light measurements were performed using a EPP2000 fibre optic spectrometer with a planar sensor (Stellarnet, http://www.stellarnet-inc.com/).

Microscopy analyses

In all microscopy studies, leaf samples were excised from the largest fully expanded rosette leaf when plants in low R:FR showed a 10-mm bolt. Rectangular sections extending from the midvein to the leaf edge were cut from the middle part of each leaf, fixed, dehydrated and embedded in Spurr's resin following standard procedures. For light microscopy, 0.5-μm sections were stained with 1% toluidine blue in 1% sodium tetraborate and viewed on an Olympus BH2 microscope (http://www.olympus.com/). Analysis of leaf pavement cells was performed using previously described SEM procedures (Halliday and Whitelam, 2003). Mean cell areas were measured using IMAGE J software (http://rsbweb.nih.gov/ij/). Leaf sections from three separate plants grown in each experimental condition were used for cell area measurements (n = 15).

Chlorophyll extraction

Chlorophyll contents were recorded from the largest rosette leaf at 2 dpb in low R:FR-grown lines. Chlorophyll was extracted from 5-mm^2 diameter leaf discs in 80% acetone and the absorbance of supernatants determined at 646 and 663 nm. Chlorophyll *a* and *b* contents were determined according to Lichtenthaler and Wellburn (1983). A minimum of five plants were assayed per treatment.

Soluble sugar assay

Soluble sugars were assayed from the largest rosette leaf at 2 dpb in low R:FR-grown lines, using the phenol–sulphuric acid method of Farrar (1993). Tissue samples (100 mg) were extracted in 90% ethanol (60°C for 1 h) before combining with 5% phenol and H_2SO_4. The absorbance of each sample was recorded at 485 nm and soluble sugar contents determined from a sucrose calibration curve (n = 3).

Metabolite profiling

Metabolite profiling was performed in whole rosettes at 2 dpb in low R:FR-grown lines. Tissue samples (200 mg) were extracted in 80% ethanol and analysed by GC-MS using similar procedures to Jung *et al.* (2009). Sorbitol (200 μl of a 1 mg ml^{-1} aqueous solution) was added before extraction as an internal standard to correct for differences in extraction efficiency, subsequent differences in derivatisation efficiency and changes in sample volume during heating. All peaks above a set minimum threshold were integrated, whether or not their identity was known. A large user-created database (>1200 spectra) of mass spectral electron impact fragmentation patterns of trimethylsilyl-derivatised compounds were used to identify the metabolites of interest to be quantified. Peaks were quantified by area integration and the concentrations were normalized to the quantity of the internal standard (sorbitol) recovered, amount of sample extracted, derivatised and injected. Three replicate samples were analysed per treatment and the metabolite data averaged.

Petiole length, leaf area, leaf angle, plant biomass and flowering time measurements

For leaf area and petiole length measurements, the largest fully expanded rosette leaf (leaf 7) was excised from each plant when plants grown in low R:FR showed a 10-mm bolt. Leaves were photographed and measured using IMAGE J software. Biomass measurements were performed in parallel to leaf area analyses. Whole plants were excised from the soil, roots washed, dried overnight at 70°C and weighed. Flowering times were recorded by counting rosette leaves when plants displayed a 10-mm bolt. Leaf angles were measured from the horizontal soil surface using a protractor, following 5 days in low R:FR (leaf angles <5° were beyond precise measurement so were recorded as 5°). Measurements from leaves five and six were averaged as these leaves displayed the largest leaf angles. Eight to 12 plants were measured, per response, per treatment. All experiments were repeated multiple times with similar results.

Quantitative trait locus mapping

The QTL analysis of the cool temperature SAS was performed using a L*er* × Cvi NIL population (Keurentjes *et al.*, 2007). Sixteen plants of each line were grown with L*er* and Cvi controls in high and low R:FR at 16°C as described above. Measurements of leaf area and petiole length were recorded when plants in low R:FR

displayed a 10-mm bolt. The QTL region identified in NIL 2-8 was narrowed to 1.3 Mb by comparative analysis with NILs 2-7, 2-9, 2-11, 2-13, 2-15 and 2-17. Fine mapping was achieved by crossing NIL 2-8 to its L*er* parent to create a segregating population. One hundred and fifty F_2 plants were genotyped using the markers in Table S2. All were developed using polymorphisms already described (Clark *et al.*, 2007).

Construction of *pER::*GUS reporter lines

The *ERECTA* promoter (–636 bp) was amplified from L*er* and Cvi using Gateway compatible primers (GW-attB4-pER-F-TGTATAGA AAAGTTGTCCATCTGTGAAACAAGCCACA; GW-attB1-pER-R-TTT TGTACAAACTTGGTTCTCACACACAGTCTTAAAAC) and standard Gateway cloning procedures (Invitrogen, http://www.invitrogen.com/). The PCR products were cloned into the donor vectors pDONR-P4P1R (VIB, Belgium, http://www.vib.be/en/) and pDONR221 containing *β-GLUCURONIDASE* (*uid-A*, GUS) (provided by D. Twell, University of Leicester, UK) to produce *pER-ECTA::*GUS in the destination vector pB7M24GW,3. All constructs were verified by sequencing before transformation in to *Agrobacterium tumefaciens* strain GV 3101 by the freeze–thaw method. Plants were transformed by floral dipping and basta-resistant plants selected for further analysis. The T_3 progeny were used for GUS assays.

GUS assay

GUS activity was assayed in rosettes (2 dpb in low R:FR-grown lines) and seedlings (following 4 days of low R:FR treatment). Samples were immersed in 3 ml of assay buffer containing 0.1 M $NaPO_4$ (pH 7.0), 10 mM EDTA, 0.1% Triton X-100, 1 mM $K_3Fe(CN)_6$, 2 mM 5-bromo-4-chloro-3-indolyl glucuronide salt (X-Gluc, Melford Laboratories, http://www.melford.co.uk/) at 37°C and incubated overnight in the dark. Samples were then washed multiple times in 50% ethanol and photographed.

Gene expression analysis by quantitative PCR

The RNA extraction, cDNA synthesis and quantitative (q)PCR were performed using SYBR Green detection (Sigma, http://www.sigmaaldrich.com/) as described previously (Franklin and Whitelam, 2007). Tissue was harvested from whole rosettes, 2 dpb in low R:FR-grown plants (*ATHB2*, *PIL1* and *ERECTA*) or following 24 h in low R:FR (*ERECTA*). Expression values were normalised to *ACTIN2*, using the primers ActinF (TCAGATGCCCAGAAGTGTTG TTCC) and ActinR (CCGTACAGATCCTTCCTGATATCC). *ATHB2*, *PIL1* and *ERECTA* were amplified using the primers ATHB2F (GAG-GTAGACTGCGAGTTCTTACG) ATHB2 R (GCATGT AGAACTGAG-GAGAGAGC), PIL1F (AAATTGCTCTCAGCCATTCGT GG), PIL1R (TTCTAAGTTTGAGGCGGACGCAG), ERF (ACTTGTG ATCCTTCTC ATGGTCTTAATAGC) and ERR (TCGGTGTCGAATAAGTTACTGGT TTGTC), respectively.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Cool temperature shade avoidance phenotypes in 16-h light/8-h dark cycles.

Figure S2. Early flowering occurs in low red:far-red at both temperatures.

Figure S3. *ERECTA* expression is localised to elongating tissues and cotyledons in seedlings.

Figure S4. Temperature-dependent shade avoidance in L*er*::ER and ERECTA-deficient accessions (Van-0 and Hir) with complemented lines (Van-0::ER and Hir-1::ER).

Figure S5. Hypocotyl lengths of wild type and *erecta* mutants grown in high and low red:far-red at 16°C and 22°C.

Figure S6. ERECTA regulates leaf hyponasty at 16°C and 22°C.

Figure S7. Transcript abundance of shade avoidance marker genes at 16°C and 22°C.

Table S1. Metabolite analysis of plants grown in high and low red:far-red at 16°C and 22°C.

Table S2. Primer sequences used for fine mapping long petiole quantitative trait locus.

Table S3. Polymorphisms in the Cvi *ERECTA* promoter sequence when compared with L*er*.

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