

# Ultraviolet screening increases with elevation in translucent bracts of *Rheum nobile* (Polygonaceae), an alpine ‘glasshouse’ plant from the high Himalayas

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Specialized bracts of Himalayan ‘glasshouse’ plants are well known for adapting to diverse stresses in alpine environments, thus ensuring normal sexual reproduction. However, little information is available on how such specialized plants cope with the elevational increase in stress. In this study, we determine the elevational pattern of ultraviolet (UV) protection provided by the translucent bracts of *Rheum nobile*, a giant ‘glasshouse’ plant species, endemic to the high Himalayas, and examine its effect on pollen germination and pollen tube growth. Both UV-A and UV-B radiation in the open air increased with elevation, but their intensity beneath bracts remained constant with elevation, suggesting that the bracts of *R. nobile* growing at higher elevations have an increased ability to screen UV radiation. Enhanced UV-B radiation, equivalent to that experienced at higher elevations (4800 m a.s.l.) compared to that at lower elevations (4200 m a.s.l.), significantly reduced pollen germination and pollen tube growth. Pollen grains from plants at higher elevations were not more tolerant to UV-B radiation. UV absorbance and the content of flavonoids in bracts increased with elevation, but a similar trend was not found in either UV reflectance or adaxial trichome density, suggesting that bract UV absorbance may play a more active role than bract UV reflectance in helping plants cope with the increasing UV radiation. Our results indicate that the bracts of *R. nobile* have the ability to cope with enhanced UV radiation with increasing elevation through increased UV absorbance, thus protecting pollen grains from injury caused by higher levels of UV radiation, and consequently ensuring normal sexual reproduction in stressful high-alpine conditions.

**ADDITIONAL KEYWORDS:** adaptation – flavonoids – pollen viability – UV absorbance – UV protection – UV reflectance

## INTRODUCTION

At various stages of their life cycle, plants have to cope with diverse environmental stresses, and the ability to protect sensitive structures from stresses can have a considerable effect on the distribution and abundance of species (Dalcorso, Farinati & Furini,

2010). It is therefore critical to study the patterns of plant protection along gradients of increasing stresses to understand the underlying adaptive mechanisms (McGill *et al.*, 2006). For alpine plants, ultraviolet (UV: 280–400 nm) radiation, especially UV-B (280–320 nm) radiation, is one of the most important environmental stresses (Caldwell, Robberecht & Flint, 1983; Körner, 2003). It has many direct and indirect effects on plants, including causing damage to DNA,

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proteins and membrane lipids, affecting transpiration and photosynthesis, and causing changes in growth, development and morphology (Pang & Hays, 1991; Caldwell *et al.*, 1995; Rozema *et al.*, 1997; van de Staaij *et al.*, 1997; Jansen, Gaba & Greenberg, 1998), and thus negatively influences the fitness of plants. Because UV radiation increases with elevation due to smaller optical air mass, plants from high elevations are thought to experience stronger UV radiation stress than plants at lower elevations (Körner, 2003). In response to higher UV radiation, plants living at higher elevations are expected to have a greater ability to protect themselves from UV radiation, compared to their relatives at lower elevations (Caldwell *et al.*, 1983; Körner, 2003). However, existing evidence regarding the relationship between plant protection against UV radiation and elevation is controversial. Some studies have found higher levels of UV protection at higher elevations (e.g. González *et al.*, 2002, 2007; Murai *et al.*, 2009; Peng *et al.*, 2010), whereas others have reported no such effects (e.g. van de Staaij *et al.*, 1995; Li *et al.*, 2019) or even higher levels of protection at lower elevations (e.g. Filella & Penuelas, 1999).

Among the traits that provide protection against UV radiation, UV absorbance by plant tissues has been identified as the most important mechanism (Jansen *et al.*, 1998; Iwashina *et al.*, 2004; Murai *et al.*, 2009; Barnes *et al.*, 2016). Absorbance of UV is mainly achieved by the accumulation of UV-absorbing compounds (e.g. flavonoids and related phenylpropanoid derivatives) in the epidermal layer of various organs (Caldwell, 1981; Caldwell *et al.*, 1983), thereby reducing epidermal UV transmittance (Bornman *et al.*, 2014). In addition, surface reflectance may also play an important role in protection against UV radiation. For example, in several plant species the leaf surface is able to reflect up to 70% of incoming UV radiation (Caldwell *et al.*, 1983). The magnitude of leaf UV reflectance in many plant species has been found to be related to the morphology and distribution of epicuticular structures (e.g. pubescence: Robberecht, Caldwell & Billings, 1980; Peng *et al.*, 2015). In addition to adjusting UV sunscreen protection in the epidermal layer in response to an increase in UV radiation, some plants adapt to higher UV radiation through biochemical changes in underlying tissues such as the mesophyll (Jansen *et al.*, 1998). For example, photodamaged photosystem II can be repaired by the replacement of damaged D1 protein by newly synthesized D1 (Kyle, Ohad & Arntzen, 1984). Note that any type of protective ‘strategy’ is usually obtained at a cost of resource investment and, with a fixed amount of resources, increased expenditure in resources for UV-absorbing compounds may entail reduced allocation to UV reflectance or other UV

protective traits (Bazzaz *et al.*, 1987). Thus, different plant species may adopt different UV protection strategies (Barnes *et al.*, 2016; Li *et al.*, 2019). To date, most studies on UV protection have been biased towards measuring UV protection through absorbance (e.g. McDougal & Parks, 1984; Ganzera *et al.*, 2008; Murai *et al.*, 2009; Peng *et al.*, 2010; Bernal *et al.*, 2013), ignoring reflectance or other protective traits. Furthermore, compared to the considerable attention that has been paid to the protective mechanisms in leaves, investigations of UV protection traits related to reproductive fitness are strikingly scarce (but see Zidorn, Schubert & Stuppner, 2005). In fact, vegetative and reproductive organs do not always respond in the same way to similar selection pressures (Guo *et al.*, 2012). Consequently, variation in the protective mechanisms associated with particular plant traits, plant tissues or organs has frequently been overlooked, and this may have obscured elevational patterns associated with protection against UV radiation.

Plants with highly specialized and particular morphologies, such as ‘glasshouse’ or ‘downy’ plants, are commonly found in the alpine zone of the Himalaya–Hengduan Mountain region in southwest China. It is assumed that their particular morphologies have evolved as a result of adaptation to the hostile environmental conditions in their habitat at high elevation; these include low air temperature, frequent precipitation, strong winds and intense solar radiation (Tsukaya & Tsuge, 2001). *Rheum nobile* Hook.f. & Thomson (Polygonaceae), a monocarpic perennial herb, endemic to the alpine zone of the Himalayas, has frequently been selected as a model species for investigating adaptive mechanisms in alpine environments (Zhang *et al.*, 2010; Song *et al.*, 2013b; Sun *et al.*, 2014), because of the large translucent, cream bracts concealing the towering inflorescence that measures up to 150 cm in height (Supporting Information, Fig. S1). Studies of *R. nobile* have revealed that the bracts of this species are multifunctional, maintaining warmth, screening UV radiation, protecting reproductive structures from rain, and attracting and sheltering pollinators (Terashima, Masuzawa & Ohba, 1993; Omori & Ohba, 1996, 1999; Omori, Takayama & Ohba, 2000; Zhang *et al.*, 2010; Song *et al.*, 2013b, 2014), thereby facilitating sexual reproduction in the stressful alpine habitats in which the species grows. A central concern in biology is to understand how organisms function in different environments (Körner, 2003), but the intraspecific elevational adaptive pattern associated with environmental stresses in this highly specialized plant has yet to be examined.

Due to the high atmospheric clarity, UV radiation in the Himalaya–Hengduan Mountains is particularly

high, increasing more steeply with elevation than in other regions (Cui *et al.*, 2008). Thus, studying how plants of *R. nobile* protect themselves against enhanced UV radiation with elevation can provide a better understanding of the adaptive mechanism of 'glasshouse' plants. In the study presented here, we examined the ability of bracts of *R. nobile* from different elevations to screen UV radiation and the protective effect on pollen germination and pollen tube growth. We aimed to answer the following particular questions. (1) Are pollen germination and pollen tube growth sensitive to increasing UV-B radiation, as occurs along the elevational gradient in the habitat of *R. nobile*? If so, do pollen grains from higher elevations have a higher tolerance to UV-B radiation? (2) Do bracts of *R. nobile* plants from higher elevations have an increased ability to screen UV radiation? If so, how do the UV reflection and absorbance of bracts change with elevation?

## MATERIAL AND METHODS

### STUDY SPECIES AND SITE

*Rheum nobile* is a giant perennial monocarpic herb endemic to the eastern high Himalayas, occurring at elevations ranging from 4000 to 6000 m a.s.l. and inhabiting mostly open scree (Li & Gao, 1998; Chowdhery & Agrawala, 2009). It has large rosulate leaves in the vegetative stage and flowering occurs between early June and early July at an age of *c.* 33 years (B. Song *et al.*, unpublished data). In the year of flowering, a stout conical compound raceme 1.0–1.5 m in height is produced with several thousand flowers concealed by large translucent bracts, which persist until the seeds have ripened (Song *et al.*, 2013a). The fruits mature between September and early October.

The study was conducted in the alpine zone at Huluhai, Shangri-la County, Yunnan Province, southwest China (28°31'N, 99°57'E), where the species grows from 4200 to 4800 m a.s.l. Three sampling sites for plants of *R. nobile* located at low, middle and high elevation (4200, 4500 and 4800 m a.s.l.) were selected along the same slope, and a voucher specimen (No. BS201001) was preserved in the Herbarium of Kunming Institute of Botany, Chinese Academy of Sciences (KUN). The climate of the study region is characterized by the summer monsoon. Mean annual precipitation recorded from 1982 to 1984 at the nearest meteorological station (28°23'N, 99°01'E, 4290 m a.s.l.), 95 km from the study sites, was 680–790 mm. The mean annual air temperature is –1.0 °C, and the difference in mean monthly

temperature between the coldest and the warmest month is around 15 °C (Wang, 2006).

### INTENSITY OF UV RADIATION

At each elevation, four plants were selected randomly each day between 15 and 22 June 2017 during the flowering period. Following the method of Song *et al.* (2013b), the intensity of UV-A (320–400 nm) and UV-B radiation beneath three bracts of each plant was measured at 14:00 h using UV-radiometers (Photoelectric Instrument Factory, Beijing Normal University). In addition, the intensity of UV-A and UV-B radiation in open air was measured immediately after the measurement beneath each bract. These measurements were taken by three observers at each elevation simultaneously. In total, 32 measurements for UV-A and 32 measurements for UV-B radiation in the open air or beneath the bracts were available per elevation. Each plant was treated as a replicate and, before analysis, means for each plant were calculated.

### POLLEN GERMINATION AND POLLEN TUBE GROWTH

To test the response of pollen grains to UV-B radiation exposure, pollen grains were harvested from newly opened anthers from 15 plants at each elevation. The pollen grains of each plant were cultured in six 9-cm-diameter Petri dishes, each with a solid medium containing 15% sucrose, 1.5% bacto-agar, 0.01% H<sub>3</sub>BO<sub>3</sub>, 0.03% Ca(NO<sub>3</sub>)<sub>2</sub>·4H<sub>2</sub>O, 0.02% MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.01% KNO<sub>3</sub> and 0.01 KH<sub>2</sub>PO<sub>4</sub> (Feng *et al.*, 2000). These plates (in total, 90 plates per elevation) were exposed to three treatments (in each treatment each plant was represented by two plates): artificial UV-B radiation at two different intensities (6.0 and 31 μW cm<sup>-2</sup> s<sup>-1</sup>) provided by an UV analyser (Shanghai Science and Analytical Instruments, Jihui, Shanghai), thus simulating the intensity of UV-B radiation beneath the bracts and the increased intensity of UV-B radiation in the open air at 4800 m a.s.l. as compared with 4200 m a.s.l. In addition, no UV-B radiation (0 μW cm<sup>-2</sup> s<sup>-1</sup>) was set as a third treatment (control). After 3 h of exposure, the plates were incubated under visible light for 5 h. Then, growth of pollen was halted by adding a few drops of killing and preserving solution comprising water, glycerine, formaldehyde and glycerol acetic acid (72:20:5:3, by vol.) to each plate (Feng *et al.*, 2000). At least 1000 pollen grains were scored per plate for determination of per cent germination, and 100 were selected to measure pollen tube length using a light microscope when the elongation was greater than pollen diameter (Feng *et al.*, 2000). For percentage pollen germination and pollen tube length, each plant

was considered a replicate and means per plant were calculated before statistical analysis.

#### UV RADIATION ABSORBANCE AND FLAVONOID CONTENT

To measure UV-absorbing compounds, ten plants at each elevation were randomly selected. A disc of c. 0.18 cm<sup>2</sup> was harvested from three bracts of each plant using a hole punch and immediately frozen in liquid nitrogen. These discs were extracted at 4 °C in the dark for 24 h in an acidified methanol solution (5 mL 70% methanol:29% H<sub>2</sub>O:1% HCl) (Barnes *et al.*, 2008; Li *et al.*, 2019). The absorbance of the extracts was measured using an ELISA microplate reader. The absorbance per unit area at 305 and 360 nm (Abs cm<sup>-2</sup>) was used to represent the absorbance of the UV-B and UV-A bands, respectively (Li *et al.*, 2019). Each plant was considered a replicate and thus the UV absorbance of each individual plant was the average measurement from the three bracts.

To measure the concentration of flavonoids in bracts, we collected the rest of the bracts from the same plants used for determining UV-absorbing compounds at each elevation. We chose flavonoids because they are widely recognized as important UV-absorbing substances (Caldwell *et al.*, 1983; Iwashina *et al.*, 2004). Total flavonoid content was determined following the method of Calado *et al.* (2015) with slight modifications. Bracts from each plant were finely ground in liquid nitrogen, and c. 300 mg was used for extraction with aqueous methanol (1:1, v/v); 200 µL of solution was transferred to another 10-mL flask, adding 200 µL of a 5% AlCl<sub>3</sub> solution. The flask was shaken and heated to 20 °C for 30 min, and the absorbance was measured at 425 nm (Cary 50 Spectrophotometer, Varian, Palo Alto, CA, USA). The concentration of flavonoids was calculated by using a standard curve for quercetin dehydrate (Sigma-Aldrich, St. Louis, MO, USA).

#### TRICHOME DENSITY AND REFLECTANCE OF UV RADIATION

To measure the spectral characteristics of bracts for the UV wavelengths, ten plants were selected randomly at each elevation; these plants were different from those used for the UV absorbance and flavonoid content experiments. Four bracts from each of the plants were collected and their reflectance spectra were measured relative to a highly UV-reflecting white MgSO<sub>4</sub> standard at an angle of 45°. The bracts were kept fresh until they were measured (within 3 h) using a spectroradiometer (USB Ocean Optics 2000+, Rochester, NY, USA) equipped with a Xenon Pulse X2 lamp light source, following Song *et al.*

(2015a). Three measurements were taken for each bract. The reflectance measured at 305 and 360 nm was used to represent the reflectance of the UV-B and UV-A bands, respectively (Li *et al.*, 2019). Individual plants were treated as replicates and, from repeated measurements, means per plant were calculated before statistical analysis.

To investigate trichome density on the adaxial surface, we collected another three bracts from each of the ten plants used for reflectance measurements at each elevation. Under a scanning electron microscope (Sigma 300, Zeiss, Oberkochen, Germany), trichome density was evaluated by counting trichomes from five samples per bract (a sample was a field of a standard area using a scanning electron microscope). Individual plants were treated as replicates and mean trichome density per plant was calculated before statistical analysis.

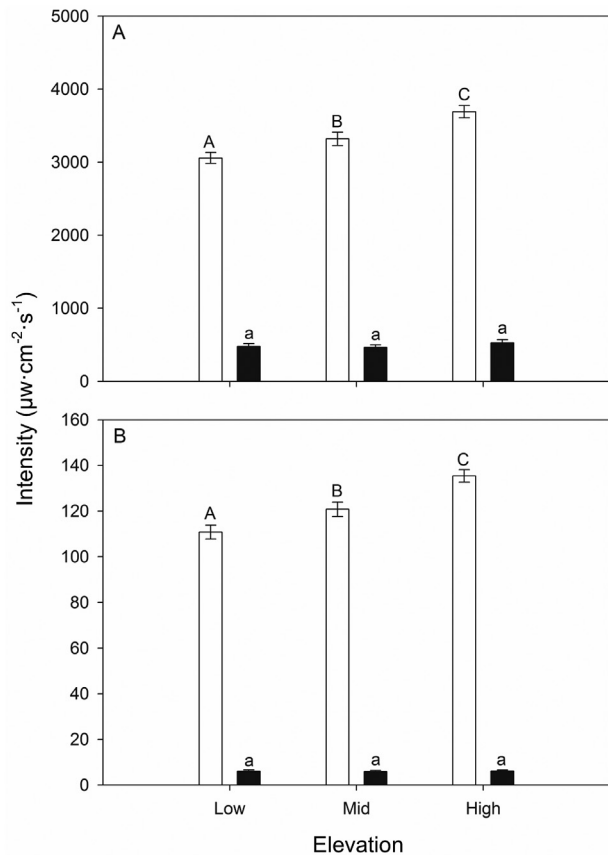
#### DATA ANALYSIS

Two-way ANOVA was used to test for the effects of bracts and elevation on the intensity of UV-A and UV-B radiation and the effect of UV-B radiation and elevation on pollen germination and pollen tube length. One-way ANOVA was used to test for the effect of elevation on the intensity of UV-A and UV-B radiation in the open air or beneath the bracts, UV reflectance, UV absorbance, concentration of flavonoids, adaxial trichome density, pollen germination and pollen tube length. Multiple comparisons of means were performed using Tukey's test at the 0.05 significance level. All analyses were performed with SPSS v.18.0. Measurements are reported as means ± 1 SE.

## RESULTS

#### INTENSITY OF UV RADIATION

Bracts significantly decreased the intensity of UV-A radiation ( $F_{1,186} = 2881.69$ ,  $P < 0.001$ ) and UV-B radiation ( $F_{1,186} = 4400.27$ ,  $P < 0.001$ ), with 85% and 95% of UV-A and UV-B radiation being screened by them, respectively. This screening effect was significantly stronger at higher elevations (UV-A:  $F_{2,186} = 10.09$ ,  $P < 0.001$ ; UV-B:  $F_{2,186} = 16.44$ ,  $P < 0.001$ ; Fig. 1). In the open air, the intensity of both UV-A and UV-B radiation increased with elevation (UV-A:  $F_{2,93} = 14.52$ ,  $P < 0.001$ ; UV-B:  $F_{2,93} = 17.14$ ,  $P < 0.001$ ). Compared with at low elevation, the intensity of UV-A and UV-B radiation increased by 20.8% and 22.7%, respectively (UV-A: 3693.9 vs. 3058.0 µW cm<sup>-2</sup> s<sup>-1</sup>; UV-B: 135.4 vs. 110.8 µW cm<sup>-2</sup> s<sup>-1</sup>; Fig. 1). However, the intensity of UV-A and UV-B radiation beneath bracts did not change across elevations (UV-A:

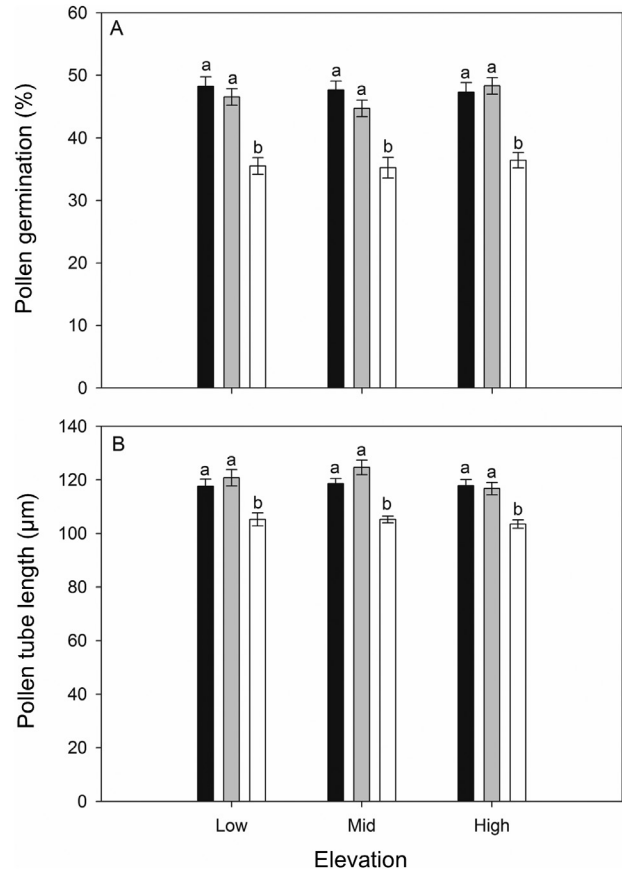


**Figure 1.** Intensity of UV-A (A) and UV-B (B) radiation in open air (white bars) and beneath bracts of *Rheum nobile* (dark grey bars) at low (4200 m), mid- (4500 m) and high (4800 m) elevation in Huluhai, Yunnan, south-west China. Data are shown as means  $\pm$  SE ( $n = 32$  per elevation). Different letters denote significant differences at  $P < 0.05$ .

$F_{2,93} = 0.73$ ,  $P = 0.49$ ; UV-B:  $F_{2,93} = 0.05$ ,  $P = 0.95$ ; UV-A:  $490.0 \mu\text{W cm}^{-2} \text{s}^{-1}$ ; UV-B:  $6.0 \mu\text{W cm}^{-2} \text{s}^{-1}$ ; Fig. 1).

#### POLLEN GERMINATION AND POLLEN TUBE GROWTH

Pollen grains collected from different elevations did not differ significantly in either per cent pollen germination or pollen tube growth across different UV-B intensities (per cent pollen germination:  $F_{2,126} = 0.84$ ,  $P = 0.43$ ; pollen tube length:  $F_{2,126} = 1.90$ ,  $P = 0.15$ ; Fig. 2). However, UV-B radiation intensity had a significant effect on per cent pollen germination and pollen tube growth (per cent pollen germination:  $F_{2,126} = 66.54$ ,  $P < 0.001$ ; pollen tube length:  $F_{2,126} = 46.09$ ,  $P < 0.001$ ; Fig. 2). When the data were pooled across elevations, pollen grains exposed to UV-B radiation of  $6.0 \mu\text{W cm}^{-2} \text{s}^{-1}$  had a germination rate of  $46.5 \pm 0.8\%$  and a pollen tube length of  $120.7 \pm 1.5 \mu\text{m}$ ; neither was significantly different from pollen grains



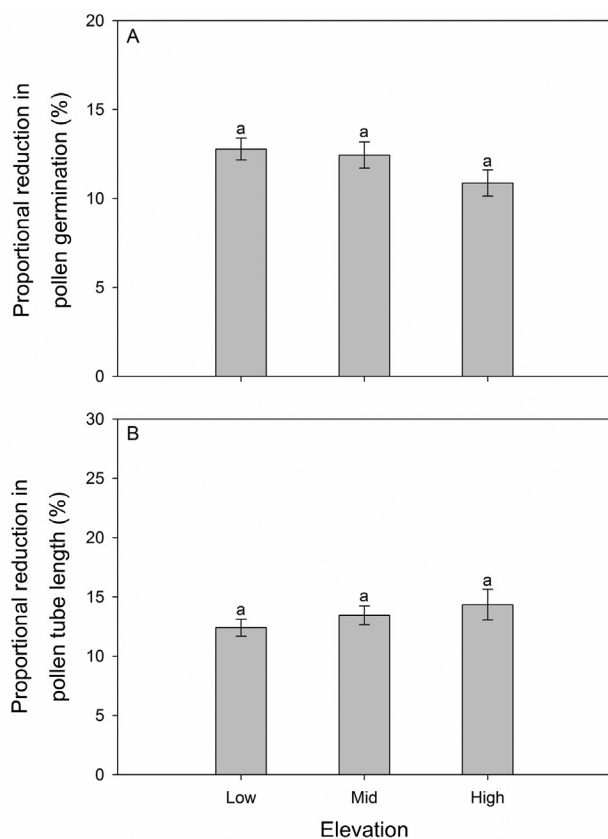
**Figure 2.** Per cent pollen germination (A) and pollen tube length (B) of pollen grains of *Rheum nobile* collected at low (4200 m), mid- (4500 m) and high (4800 m) elevation in Huluhai, Yunnan, south-west China. Pollen was exposed to different intensities of UV-B radiation:  $0 \mu\text{W cm}^{-2} \text{s}^{-1}$  (control, dark grey bars),  $6.0 \mu\text{W cm}^{-2} \text{s}^{-1}$  (light grey bars) and  $31.0 \mu\text{W cm}^{-2} \text{s}^{-1}$  (white bars). Data are shown as means  $\pm$  SE ( $n = 15$  per elevation). Different letters denote significant differences at  $P < 0.05$ .

exposed to the control treatment (with no UV-B radiation: per cent pollen germination was  $47.7 \pm 0.8\%$ , and pollen tube length was  $118.1 \pm 1.3 \mu\text{m}$ ). In contrast, pollen grains exposed to UV-B radiation of  $31 \mu\text{W cm}^{-2} \text{s}^{-1}$  were negatively affected compared to the treatments reported above (pollen germination:  $35.7 \pm 0.8\%$ ,  $F_{2,132} = 67.58$ ,  $P < 0.001$ ; pollen tube length:  $104.7 \pm 1.0 \mu\text{m}$ ;  $F_{2,132} = 45.74$ ,  $P < 0.001$ ).

The reduction in pollen germination rate and pollen tube growth when pollen grains were exposed to UV-B radiation of  $31 \mu\text{W cm}^{-2} \text{s}^{-1}$  compared to the control (no UV-B radiation) did not depend on the elevational origin of the pollen. ANOVA results were as follows – reduction in pollen germination:  $F_{2,42} = 2.10$ ,  $P = 0.14$ ; reduction in pollen tube growth:  $F_{2,42} = 1.02$ ,  $P = 0.37$  (Fig. 3). This indicates that pollen grains from higher elevations had no greater tolerance to UV radiation.

## FLAVONOID CONTENT AND ABSORBANCE OF UV RADIATION

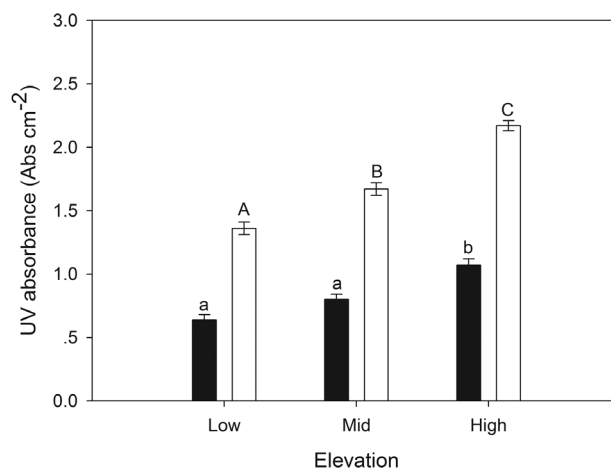
At all elevations, the indicative UV-B absorbance of bracts (measured per unit area at 305 nm) was 2.1 times greater than indicative UV-A absorbance (measured at 360 nm). The ability of bracts to absorb UV radiation was significantly affected by elevation for both UV-A and UV-B (UV-A:  $F_{2,27} = 23.36$ ,  $P < 0.001$ ; UV-B:  $F_{2,27} = 80.46$ ,  $P < 0.001$ ; Fig. 4). Compared with bracts from low elevation, UV-A and UV-B absorbance by bracts from high elevation was increased by 67.2% and 60.0%, respectively. Similarly, elevation had a significant effect on the concentration of flavonoids in bracts ( $F_{2,27} = 18.47$ ,  $P < 0.001$ ; Fig. 5). Bracts of plants from 4800 m a.s.l. had the highest concentration of flavonoids ( $25.9 \pm 0.91$  mg), followed by bracts of plants from 4500 m a.s.l. ( $21.8 \pm 0.70$  mg), and then bracts of plants from 4200 m a.s.l. ( $18.8 \pm 0.85$  mg).



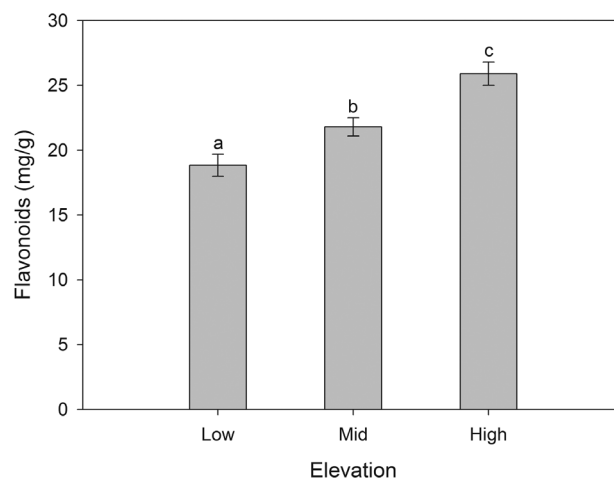
**Figure 3.** Proportional reduction in per cent pollen germination (A) and pollen tube length (B) of pollen grains of *Rheum nobile* from low (4200 m), mid- (4500 m) and high (4800 m) elevation in Huluhai, Yunnan, south-west China, after exposure to UV-B radiation of  $31 \mu\text{W cm}^{-2} \text{s}^{-1}$  relative to the control ( $0 \mu\text{W cm}^{-2} \text{s}^{-1}$ ). Data are shown as means  $\pm$  SE ( $n = 15$  per elevation).

## TRICHOME DENSITY AND REFLECTANCE OF UV RADIATION

Bracts from different elevations did not differ significantly with respect to either UV-A or UV-B reflectance (UV-A:  $F_{2,27} = 0.25$ ,  $P = 0.79$ ; UV-B:  $F_{2,27} = 0.35$ ,  $P = 0.71$ ; Supporting Information, Fig. S2). On average, bracts reflected 1.4% and 1.6% of UV-A and UV-B, respectively. Similarly, no significant elevational



**Figure 4.** UV-A and UV-B absorbance of bracts in *Rheum nobile* from low (4200 m), mid- (4500 m) and high (4800 m) elevation in Huluhai, Yunnan, south-west China. Data are shown as means  $\pm$  SE ( $n = 10$  per elevation). The dark grey bars indicate UV-A reflectance at 360 nm and the white bars indicate UV-B reflectance at 305 nm. Different letters denote significant differences at  $P < 0.05$ .



**Figure 5.** Concentration of flavonoids in bracts of *Rheum nobile* from low (4200 m), mid- (4500 m) and high (4800 m) elevation in Huluhai, Yunnan, south-west China. Data are shown as means  $\pm$  SE ( $n = 10$  per elevation). Different letters denote significant differences at  $P < 0.05$ .

variation was found for the adaxial trichome density of bracts ( $F_{2,27} = 1.59$ ,  $P = 0.22$ ; Fig. S3).

## DISCUSSION

The results from this study indicate that the showy bracts of *R. nobile* have an increased ability to screen UV radiation when originating from sites at high elevation where they are exposed to higher levels of UV radiation, thereby protecting the pollen grains within from harmful UV radiation. Bract UV absorbance exhibited a significant increase with increasing elevation, but no significant elevational variation in bract UV reflectance was found. There was no significant elevational variation in the sensitivity of pollen grains to UV radiation. These results suggest that *R. nobile* can cope with the increased UV radiation at higher elevations through an adjustment of bract UV absorbance to protect pollen grains, thus facilitating normal sexual reproduction in stressful high-alpine habitats.

Substantial elevational variations in UV-A and UV-B radiation were observed at our study sites, with an increase of up to 34.6% and 37.9% per 1000-m elevational increase on sunny days for UV-A and UV-B, respectively. Clearly, the increase in UV radiation with elevation in the Hengduan Mountains is more pronounced than in other regions (Seckmeyer & McKenzie, 1992; Blumthaler, Ambach & Ellinger, 1997; Schmucki & Philipona, 2002; Körner, 2003). UV radiation can damage pollen DNA, injure membranes and lead to differential transmission of alleles at various loci, resulting in decreased pollen viability (Torabinejad *et al.*, 1998; Feng *et al.*, 2000). Our pollen germination experiment showed that enhanced UV-B radiation could significantly reduce both pollen germination and pollen tube growth, with a  $10 \mu\text{W cm}^{-2} \text{s}^{-1}$  increase in UV-B radiation causing a 10% and 5% decrease in pollen germination and pollen tube length, respectively. This suggests that pollen grains of *R. nobile* are highly sensitive to UV-B radiation. Although we did not determine directly the reproductive fitness of *R. nobile*, reduced pollen germination and pollen tube growth resulting from elevated UV-B radiation led to lower fertilization rates (Zelles, Seibold & Ernst, 1977; Musil, 1995; Song *et al.*, 2013b), consequently negatively affecting seed production. This indicates that plants from higher elevations experience a stronger pressure to adjust to harmful UV radiation. Our field investigation found no significant difference in the intensity of either UV-A or UV-B radiation beneath bracts at the different elevations considered. This lack of elevational variation in UV radiation beneath bracts, together with the higher intensity of UV radiation in open air at higher

elevations, suggests that the bracts of *R. nobile* plants are able to adjust their UV protection to account for enhanced levels of UV radiation. Although UV radiation is widely recognized to be biologically harmful, bracts of *R. nobile* did not filter all UV radiation, as has been reported in other protective structures (e.g. corollas) of other alpine plants (e.g. Chen *et al.*, 2013; Gao *et al.*, 2019). Furthermore, the amount of UV radiation that was allowed to penetrate the bracts is almost identical across different elevations. The causes of these merit attention in future studies. In addition to pollen grains, the gynoecium (including the fertilized ovules) of *R. nobile* may also be sensitive to increased UV radiation, because intense UV radiation is harmful to apical meristems during cell division and cell differentiation (Musil, 1994, 1995; Harley *et al.*, 1996). Therefore, maintaining a high level of sexual reproduction may have been an important selective factor in the evolution of a protective adjustment capacity to high UV radiation by the bracts of *R. nobile*. Genetic differentiation associated with stress tolerance has been demonstrated in many species (e.g. Keeley, 1979; Biswas & Jansen, 2012). However, from our experimental design we cannot know whether, or to what a degree, the observed adjustment in UV screening at high elevation by the bracts of *R. nobile* is due to intraspecific genetic variation.

It is widely acknowledged that the stress resistance of pollen grains is higher in plants that are more likely to be exposed to stress (Jones, 1967; Sullivan, Teramura & Ziska, 1992). In our study, however, pollen grains collected at different elevations did not differ in response to elevated UV-B radiation, suggesting that pollen grains of plants of *R. nobile* growing at higher elevations did not have higher resistance to UV radiation. It has been suggested that the capacity of pollen to resist UV radiation depends mainly on the accumulation of UV-absorbing compounds in the sporoderm (Caldwell *et al.*, 1983; Feng *et al.*, 2000). However, for plants to synthesize these compounds involves great resource costs (Guidi *et al.*, 2011). In *R. nobile*, an increasing allocation to UV resistance in pollen grains is not favoured, probably because the multifunctional protective bracts already allocate more resources to UV screening at higher elevations.

Both UV reflectance and UV absorbance are important ways by which terrestrial plants cope with UV radiation (Caldwell *et al.*, 1983; Körner, 2003). In our study, bracts showed a significant increase in UV absorbance with increasing elevation, whereas no significant change in UV reflectance was found. This result suggests that, in *R. nobile*, the increasing protection against UV radiation with elevation conferred by the bracts is mediated mainly through absorbance not reflectance; this is consistent with many previous studies focusing on leaves (Caldwell,

1971; Gausman, Rodriguez & Escobar, 1975; Caldwell *et al.*, 1983; Filella & Penuelas, 1999; Körner, 2003). Iwashina *et al.* (2004) reported that high levels of flavonoids occur in the bracts of *R. nobile* and these compounds have been widely confirmed to be effective in absorbing UV radiation (Bidel *et al.*, 2007; Barnes *et al.*, 2016). Similarly, in our study, high levels of flavonoids were detected in the bracts of *R. nobile*, with bracts from plants at higher elevations having higher concentrations of flavonoids. Thus, the fact that the ability of bracts to absorb UV radiation increases with elevation may not be surprising. Some recent studies have found that different flavonoid compounds do not necessarily respond in the same way to similar UV radiation exposure (e.g. Neugart *et al.*, 2012; Barnes *et al.*, 2016). However, we did not determine precisely the content of different flavonoid compounds. Thus, further studies should be conducted to test if the responses to changing elevations is consistent across all classes of flavonoid compounds in the bracts of *R. nobile*. Elevational variation in flavonoids may also have consequences for variations in plant responses to other stresses (e.g. herbivory), for pollinator attraction and leaf protection against damage caused by low temperatures (Harborne & Grayer, 1993; Bilger, Rolland & Nybakken, 2007; Abdala-Roberts *et al.*, 2016). Thus, additional studies examining how UV protection interacts with other ecological and physiological functions should be undertaken to gain a full understanding of the evolutionary processes leading to the increase in UV absorbance with elevation. Nevertheless, our results indicate that the elevational adjustment capacity with respect to the UV absorbance by bracts of *R. nobile* was probably selected for, at least in part, to protect pollen grains and ensure normal sexual reproduction at higher elevations with higher levels of UV radiation.

Previous studies have found that leaf UV reflectance in many plant species is explained largely by epicuticular structures, such as intense glaucousness (Clark & Lister, 1975; Mulroy, 1979) and dense pubescence (Robberecht *et al.*, 1980; Peng *et al.*, 2015). However, in *R. nobile* only a few trichomes were found on the adaxial surface of the bracts; furthermore, trichome density did not vary with elevation. This may partly explain the lower mean UV reflectance across the entire gradient relative to the leaves of other plants (Caldwell, 1971; Gausman *et al.*, 1975; Li *et al.*, 2019) and the lack of a significant elevational gradient in UV reflectance. Indeed, UV reflectance through epicuticular structures has been found not to be wavelength-selective. For example, leaf pubescence and glaucousness with high UV reflectance exhibit approximately the same reflectance in the photosynthetically active (PA) and infrared (IR) range (Clark & Lister, 1975; Caldwell *et al.*, 1983). It should

be noted that, in addition to UV radiation, stress from low temperature is also increased with elevation (Körner, 2003). Accordingly, bracts of *R. nobile* at higher elevations experience a stronger selection pressure for warming interior reproductive organs. Such a warming effect by bracts depends to a large extent on the transmitted PA and IR range (Omori *et al.*, 2000; Song *et al.*, 2013b). Flavonoids in the bracts, unlike surface structures, can selectively filter sunlight, thereby absorbing much of the potentially deleterious UV radiation while transmitting PA and IR radiation (Caldwell *et al.*, 1983; Day, Vogelmann & DeLuci, 1992; Omori *et al.*, 2000). Thus, it is not surprising that bracts of *R. nobile* attenuated higher levels of UV radiation by enhancing UV absorbance rather than higher UV reflectance with increasing elevation.

In conclusion, our results reveal that the bracts of *R. nobile* plants from higher elevations have a better ability to screen UV radiation and thereby prevent pollen grains from being injured as this radiation increases. Our results also demonstrate that bract UV absorbance in association with increasing flavonoids plays a more active role in protecting plants from increasing UV radiation as compared with bract UV reflectance. Although several studies have tested how 'glasshouse' plants adjust to intense UV radiation in alpine environments and have found that the translucent bracts play an important role in protecting against high UV radiation (Omori *et al.*, 2000; Tsukaya, 2002; Yang & Sun, 2009; Zhang *et al.*, 2010; Song *et al.*, 2013b, 2015b), our study provides the first direct evidence of an elevational pattern of UV protection enhancing reproductive fitness. Further studies on how these plants adjust to changes in environmental factors such as temperature, precipitation and pollinator abundance with elevation, and how much such variation is due to intraspecific genetic variation, should be undertaken to provide a better understanding of the survival strategies of plants in harsh alpine habitats.

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#### AUTHOR CONTRIBUTIONS

H.S. and B.S. conceived and designed the experiments. B.S., Y.Q.G., M.S.S. and L.S. conducted the experiments. B.S. analysed the data and wrote the manuscript. J.S. was consulted about data analyses and revised the manuscript.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher’s website.

**Figure S1.** A flowering plant of *Rheum nobile* in Huluhai, Yunnan, south-west China.

**Figure S2.** Reflectance of UV-A and UV-B radiation from the adaxial surface of bracts of *Rheum nobile* plants at low (4200 m), middle (4500 m) and high (4800 m) elevation in Huluhai, Yunnan, south-west China. The dark grey bars indicate UV-A reflectance at 360 nm and the white bars indicate UV-B reflectance at 305 nm. Data are shown as means  $\pm$  SE ( $n = 10$  per elevation).

**Figure S3.** Adaxial trichome density of bracts of *Rheum nobile* at low (4200 m), middle (4500 m) and high (4800 m) elevation in Huluhai, Yunnan, south-west China. Data are shown as mean  $\pm$  SE ( $n = 10$  per elevation).