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Selection for resistance to white pine blister rust affects the abiotic stress tolerances of limber pine

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ABSTRACT

Limber pine (*Pinus flexilis*) mortality is increasing across the West as a result of the combined stresses of white pine blister rust (*Cronartium ribicola*; WPBR), mountain pine beetle (*Dendroctonus ponderosae*), and dwarf mistletoe (*Arceuthobium cyanocarpum*) in a changing climate. With the continued spread of WPBR, extensive mortality will continue with strong selection against trees that lack genetic resistance to the disease. Naturally-occurring resistance to the non-native fungal pathogen *C. ribicola* is present in limber pine and is the cornerstone of restoration strategies. Disease resistance to native pathogens can carry a strong fitness cost to the host in the absence of the pathogen. However we suspect this to be unlikely in the case of resistance to a non-native pathogen as the resistance would not have persisted in the pre-invasion population. Genetic resistance to a novel stress which the species has not co-evolved may be neutral or carry a benefit to the host via a function that offers adaptive benefit for environmental factor(s), biotic or abiotic, under which it did evolve. Because plant disease defenses can share physiological activity with those that mediate freezing and drought stress sensitivity, both stresses for which limber pine is responsive, we compared cold and drought tolerances of limber pine seedling families from trees previously determined to have (R families) and not have (S families) the *Cr4* allele for qualitative resistance to WPBR. R families constitutively had (1) greater cold hardiness than S families and (2) lower stomatal conductance than S families during moderate drought, suggesting that R and S families have different abiotic stress responses such that the post-invasion populations may have the potential for modified competitive ability, especially under a warming climate. The presence of different stress tolerances in R families may also inform hypotheses to explain the presence and frequency of a resistance gene against a non-native pathogen to which the species was not previously exposed. We conclude that as the frequency of qualitative resistance to WPBR increases, through natural selection or planting of disease resistant seedling stock, the resultant populations may have a different suite of stress tolerance traits than pre-invasion populations. The shift in the fundamental niche of limber pine in the presence of *C. ribicola* should be considered when selecting seed sources and habitats for restoration and projecting future distributions in a changing climate.

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1. Introduction

Since its introduction to western North America in 1910, the invasive fungal pathogen *Cronartium ribicola*, cause of the lethal disease white pine blister rust (WPBR), has penetrated white pine communities across the western United States and Canada and continues to spread, threatening the remaining uninfected communities (Burns et al., 2008). WPBR devastated commercial cultivation of western white pine (*Pinus monticola*) and sugar pine (*Pinus lambertiana*) (Kinloch et al., 2008) and is a major contributor to substantial declines in other white pine communities across western North America (Tomback and Achuff, 2010). Several of the affected species serve important roles in their native ranges. Among these is limber pine (*Pinus flexilis*), which has a broad range extending from the Rocky Mountains to the eastern Sierra Nevada and eastern Oregon and an elevation range from 870 to 3500 m across its full distribution (Schoettle and Rochelle, 2000; Steele, 1990). Limber pine is an important high elevation species in the Southern Rocky Mountains, often defining the alpine treeline (Schoettle, 2004; Schoettle and Rochelle, 2000). Particularly in these high elevation communities, limber pine constitutes a keystone species through its role in stabilizing otherwise dry, unoccupied

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slopes; as an early colonizer post-fire; as a nurse plant for late-successional tree species; and as habitat and food for animal species, particularly Clark's nutcracker (*Nucifraga columbiana*), an important seed dispersal agent (see Schoettle, 2004).

In the US and Canada, limber pine is under considerable threat of future declines due to the combined impacts of WPBR, mountain pine beetle (*Dendroctonus ponderosae*), dwarf mistletoe (*Arceuthobium cyanocarpum*), and climate change and is therefore of conservation concern. Basal area losses in limber pine are estimated to exceed 40% over the next 15 years (Krist et al., 2014) and the species distribution is expected to contract under climate change in some areas (Monahan et al., 2013). Consequently, limber is listed as a Special Status Species on Bureau of Land Management land in Wyoming (USDI BLM Wyoming, 2013) and as endangered in Alberta (Government of Alberta, 2010); it is recommended for endangered species status nationally in Canada under the Species at Risk Act (Government of Canada, 2014).

Management strategies to reduce the impact of WPBR capitalize on existing genetic resistance to the disease and increasing the frequency of resistance on the landscape to sustain population numbers and restore resilience (Alberta Whitebark and Limber Pine Recovery Team, 2014; Burns et al., 2008; Keane et al., 2012; King et al., 2010; Schoettle and Sniezko, 2007). Despite its being an exotic pathogen, all North American white pine species, including limber pine, exhibit some degree of natural resistance to *C. ribicola* colonization. Breeding efforts have been in place since the 1940s to propagate families of commercial white pine species with heritable resistance mechanisms as a means of restoring devastated communities and plantations (King et al., 2010). Also underway are efforts in several non-commercial species to seed or plant still-extant populations with disease-resistant individuals to stave off potential losses in threatened communities before they occur (Burns et al., 2008; Keane et al., 2012; Keane and Schoettle, 2011; Schoettle and Sniezko, 2007).

Understanding the genetic consequences of management practices and of natural selection by novel stresses is essential for sustaining and conserving forest ecosystems (Alfaro et al., 2014; Ratnam et al., 2014). Possible correlations and trade-offs between disease resistance and plant tolerance to abiotic stress is an important but generally understudied facet of forest management (Sthultz et al., 2009). If present in the WPBR pathosystem, such correlations may influence plant performance and survival across different environments after the selective agency of *C. ribicola* invasion has reduced the frequency of susceptible individuals from the population or after managers have selectively propagated and planted seedlings exhibiting resistance, both of which could inadvertently select for or against associated adaptive traits. Artificial selection for WPBR resistance in western white pine has caused loss of rare alleles as measured with molecular markers assumed to be selectively neutral (Kim et al., 2003), yet the effect of selection on stress tolerance traits is unclear. An understanding of adaptive traits correlated with resistance (or susceptibility) will be necessary to recognize possible WPBR-induced shifts in the fundamental niche of pine species and to inform decisions about seed sources and habitats for restoration planting and how plant performance may change under future climate regimes (Chmura et al., 2011).

Geographic variation in disease resistance among populations suggests possible genetic or physiological correlations between resistance and abiotic stress tolerance. For example, quantitative resistance to WPBR in the interior west tends to increase in whitebark pine (*Pinus albicaulis*) from Montana and Idaho westward (Mahalovich et al., 2006). There is also geographic variation in qualitative resistance to WPBR, which is inherited via a single dominant gene and is characterized by a gene-for-gene hypersensitive-like reaction conditioned by an *R* gene – the *Cr* gene in the case of the WPBR-pine pathosystem. Geographic variation in the frequency of *Cr* alleles has been found in three North American white pines: sugar pine (*Cr1*), western white pine (*Cr2*) and limber pine (*Cr4*). While the average frequency of *Cr2*, *Cr1* and *Cr4* over large areas is 0.1% (range wide), 2.2% (range wide), and 5.0% (in the Southern Rockies), respectively, some populations or entire sections of the distribution of each species lack the allele altogether while others can reach frequencies of 0.8%, 8.9% and 13.8%, respectively (Kinloch, 1992; Kinloch et al., 2003; Schoettle et al., 2014). Specifically, the frequency of *Cr1* in sugar pine and *Cr2* in western white pine is much greater for both species in the high elevations of the southern Sierra Nevada than in the lower elevations of the northern Sierras and the Cascades (Kinloch, 1992; Kinloch et al., 2003; Kitzmiller, 2004), potentially suggesting an association of *Cr* genes with climate and/or abiotic stress tolerance in these species. Recent research suggests that the *Cr4* allele in limber pine also varies geographically, as it has not been detected in bulk lots and families sampled from outside the Southern Rocky Mountains (Kinloch and Dupper, 2002; Schoettle et al., 2014).

R genes to native pathogens can carry a strong fitness cost to the host in the absence of the pathogen (Tian et al., 2003); this would be very unlikely in the case of *R* genes to a non-native pathogen or the *R* gene would not have persisted in the pre-invasion population. It is more plausible, we hypothesize, that an *R* gene effective against a novel stress for which it has not co-evolved may be neutral or carry a benefit to the host possibly via a function that offers adaptive advantage to an environmental factor, biotic or abiotic, under which the host did evolve. As a result, a correlation with abiotic stress tolerance may also inform hypotheses about how *Cr* alleles have been maintained in species that have not been exposed previously to the corresponding pathogen.

Some associations between insect or disease resistance and stress tolerance have been shown in other species (Sthultz et al., 2009) and are suspected in white pines. For example, some pathogenesis-related or "PR" proteins that are involved in quantitative resistance to *C. ribicola* are known to be expressed in plants in response to abiotic stresses (Davidson and Ekramoddoullah, 1997; Ekramoddoullah and Tan, 1998). In other plants, PR proteins have exhibited the ability to suppress propagation of ice crystals *in vitro* (Zamani et al., 2003; Cabello et al., 2012) and, when overexpressed *in vivo*, to reduce frost damage and increase thermal hysteresis in whole plants (Griffith and Yaish, 2004; Liu et al., 2013). As well, endogenous induction of PR protein expression during fall cold hardening has been shown to enhance subsequent plant disease resistance and cold tolerance (Cabello et al., 2012) and PR proteins are also expressed in maritime pine (*Pinus pinaster*) (Dubos and Plomion, 2001) and winter wheat (Yeh et al., 2000) in response to drought. The role of PR proteins in both disease resistance and stress tolerance provides a basis to hypothesize an association between qualitative WPBR resistance and cold and drought tolerances in white pine species.

As a species that grows in dry rocky habitats across a range of elevations, shifts in cold and drought tolerances that may result from WPBR resistance selection may affect limber pine performance, especially in a changing climate. Limber pine exhibits conservative water-use traits (Letts et al., 2009) yet seasonal moisture stress still imposes a strong limitation to limber pine survival and is likely to be exacerbated by climate change (Millar et al., 2007; Moyes et al., 2013). Regional warming has already contributed to an increase in western U.S. tree mortality (van Mantgem and Stephenson, 2007) and in the Southern Rocky Mountains, temperatures have risen 0.5–1 °C over the last 30 years, with higher elevations warming more quickly in some areas (McWethy et al., 2010). A projected annual mean temperature increase of 2 °C is expected by 2050 with a decrease in the amount and seasonality of precipitation (Krist et al., 2014; McWethy et al., 2010). Without consideration for WPBR

selection, future climatic projections indicate a shift in suitable limber pine habitat toward higher elevations and a more patchy distribution in northern Colorado (Monahan et al., 2013). Further understanding of potential shifts in the cold and drought tolerance of post WPBR selection limber pine populations may affect those projections as well as safe seed transfer distances (O'Neill et al., 2014) for successful restoration planting efforts.

We hypothesized that correlations exist between qualitative resistance and abiotic stress tolerance in limber pine. To test this hypothesis, we sampled seed and grew seedling families from known rust-resistant (R) and rust-susceptible (S) open-pollinated seed trees from each of six different sites across a range of elevations in the Southern Rocky Mountains in a common garden. This pairing of seedling families from R and S seed trees from the same site reduce potential geographical trait variation not associated with resistance to better assess differences in just those traits correlated with the *Cr4* resistance allele. The families of seedlings from R seed trees are referred to here as "R families" and from S seed trees as "S families." Seedlings were raised for three years in a common garden and subjected to two years of fall cold tolerance testing as well as summer drought response testing. We predicted that R families would exhibit greater cold hardiness than S families and differential response to drought than S families. Because some cellular mechanisms of plant drought tolerance and cold tolerance are related (Seki et al., 2003), we also predicted that experiencing drought and recovery would enhance cold hardening during the following winter. Finally, we also tested the effect of inoculation of limber pine seedlings with *C. ribicola* on their subsequent cold hardiness the following winter. In both the cases of drought and inoculation, we predicted that these stresses would lead to greater cold hardiness of limber pine seedlings during subsequent winter cold hardiness testing.

2. Materials and methods

2.1. Plant material and seed sources

Seed from 10 open-pollinated seed trees previously shown to contain the dominant *Cr4* allele (R families) paired with 10 shown to lack it (S families) from the same sites (Table 1, Fig. 1) (Schoettle et al., 2014) were selected for the study. Sites that contained known families with and without *Cr4* resistance from as broad an elevation range as possible were included; at this time no R families have been detected from the few sites tested below 2350 m elevation. The paired family design within sites offered control for genetic background and geography, the 10 families per phenotype provided good control for detection of trait differences, and the ample number of seedlings within each family offered a strong characterization of each family. It is notable that not all individuals in each R family possess the *Cr4* allele; they are open-pollinated progeny of a *Cr4cr4* seed tree and approximately 50% of the seedlings in each R family did not inherit the *Cr4* allele and are rust-susceptible (Table 2), which would dilute the impact of resistant individuals on the observed trends, therefore making the reported correlations conservative.

Table 1
Geographical characteristics of sites of origin of limber pine families in this study.

Site ID	Elevation (m)	Latitude[a]	Longitude[a]
CH	2450	40.96908	−105.52701
CP	3120	40.65250	−105.65515
EMPN6	2650	41.25880	−105.43317
JEN	3325	39.93371	−105.65872
MCC	2370	41.18431	−105.30693
PHA	2660	41.26715	−105.43375

[a] Datum: WGS84.

Seeds were cold stratified for 60 days, and then sown into plastic boxes in a growth chamber (25 °C, 100% RH) in April, 2010 at USDA Forest Service Dorena Genetic Resource Center (DGRC; Cottage Grove, OR). Upon radicle emergence, seedlings were transplanted into Ray Leach cone-tainers (164 cm^3; Stuewe and Sons, Inc., Tangent, OR) and moved to the greenhouse. Germination and transplantation continued until the desired sample size was reached (15–20 days). A randomized complete block design was utilized. Eighteen seedlings per family were randomly assigned to each of three blocks ($N = 54$ total seedlings per family) and further separated within each block into rows of 6 seedlings that were randomly assigned a location within the block. In early spring 2012, seedlings were transplanted to 2310 cm^3 "Short One" treepots (Stuewe and Sons, Tangent, OR) and moved outside at DGRC. During the summer season plants were watered regularly.

2.2. Drought response

A subset of limber pine seedlings was selected for inclusion in a drought response experiment in the summer and fall of 2013. This included 100 plants in the drought treatment and 80 plants in the non-droughted control treatment, distributed evenly among the 10 R and 10 S families and selected at random from the common garden at DGRC. The water withholding treatment extended from August 23 (Day 1) until October 26, 2013 (Day 65). Plants were covered nightly and during rain events with multiple tarps to exclude rainfall; the efficacy of this system was monitored by measuring soil volumetric water content for all plants on a regular basis – particularly after rainfall events – using a HydroSense soil moisture probe (Campbell Scientific, Inc., Logan UT). Non-droughted control plants were hand-watered four times weekly. From October 1–26, all of the plants were moved into an open-air greenhouse due to increased ambient rainfall.

During the course of the experiment, we monitored the progression and effects of the drought treatment. This included regular measurements of pre-dawn fascicle water potentials on a subset of 20 droughted and 20 non-droughted plants, one from every family, using a pressure chamber (Model 600, PMS Instruments, Albany, OR). We also regularly measured pot weights of all plants to evaluate the progression of the drought treatment. To assess damage during drought, we regularly measured chlorophyll fluorescence as the dark-adapted F_v/F_m ratio (Maxwell and Johnson, 2000) of fascicles of all plants using an OS-5 modulated chlorophyll fluorometer (Opti-Sciences, Inc., Hudson, NH) with a modulation intensity of 60, a detector gain of 70 and saturation pulse intensity and duration of 110 and 0.8 s, respectively. Dark-adapted F_v/F_m represents the maximum potential quantum efficiency of Photosystem II with depressed values indicating plant stress and damage to Photosystem II. To measure physiological response to soil drying we measured parameters of photosynthetic gas exchange using an LI-6400 Portable Photosynthesis System (Li-Cor, Inc., Lincoln, NE). We used the same fascicles for each measurement of gas exchange, marking them at the base with a piece of string and determining total leaf surface area at the end of the experiment by measuring fascicle lengths and diameters, the latter with Mitutoyo Digimatic Calipers (Mitutoyo America Corp., Aurora, IL). Leaf cuvette conditions were maintained at a temperature of 25 °C, reference CO_2 concentration of 400 μmol mol^{-1}, PPFD of 1800 μE m^{-2} s^{-1} and leaf-to-air vapor pressure difference of 1.6 kPa. Measurements were taken between 0800 and 1400.

2.3. Cold hardiness

To assess the cold hardiness of R and S limber pine families, we sampled fascicles from seedlings in the common garden during November, 2012 and 2013 and subjected them to freezing tests.

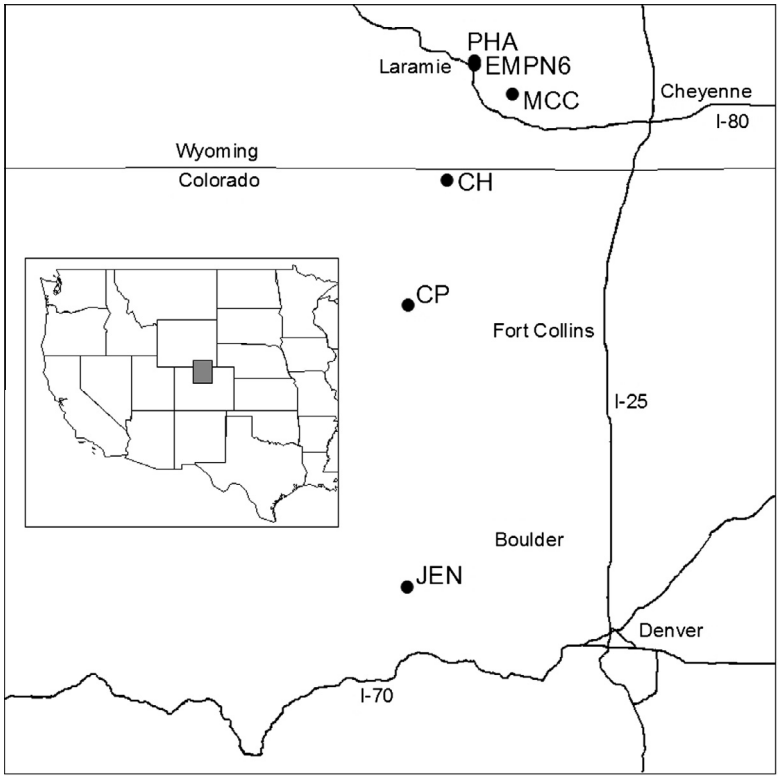

Fig. 1. Map of sampling sites in northern Colorado and southern Wyoming.

Table 2
Disease resistance phenotypes and genotypes of limber pine seed tree families in this study.

Family	Proportion of progeny displaying the resistant phenotype in previous tests[a]	Inferred seed tree genotype[a]
CH-130R	0.31[b]	*Cr4cr4*
CH-134S	0.00	*cr4cr4*
CP-137R	0.65[b]	*Cr4cr4*
CP-141R	0.41[b]	*Cr4cr4*
CP-143S	0.04	*cr4cr4*
CP-144S	0.07	*cr4cr4*
EMPN6-612R	0.54[b]	*Cr4cr4*
EMPN6-x572R	0.41[b]	*Cr4cr4*
EMPN6-x591S	0.13	*cr4cr4*
EMPN6-x595S	0.10	*cr4cr4*
JEN-148R	0.91[c]	*Cr4cr4*
JEN-150S	0.10	*cr4cr4*
JEN-154S	0.10	*cr4cr4*
JEN-155R	0.61[b]	*Cr4cr4*
MCC-123S	0.00	*cr4cr4*
MCC-124R	0.50[b]	*Cr4cr4*
PHA-107R	0.50[b]	*Cr4cr4*
PHA-110R	0.54[b]	*Cr4cr4*
PHA-113S	0.09	*cr4cr4*
PHA-114S	0.00	*cr4cr4*

[a] See Schoettle et al., 2014.
[b] Fits a 1:1 segregation ratio of diseased:stem-symptom-free.
[c] Fits a 0:1 segregation ratio of diseased:stem-symptom-free, genotype may be *Cr4Cr4*.

Two adjacent, fully-expanded, current-year fascicles were sampled from the terminal stem of each seedling. Each was taped to moist filter paper inside separate plastic sheets wrapped in aluminum foil to retain moisture and exclude light. One fascicle from each individual was assigned to the Control group, which was placed in a cold room at 3 °C until measurement, and the other to the freeze treatment. Fascicles included in the freeze treatment were chilled overnight in a Tenney TUJR-A-WF4 programmable freezer (SPX Thermal Products Solutions, New Columbia, PA) at 3 °C and then frozen at a rate of 2 °C h^{-1} to a target temperature of −34 °C; we selected this temperature after running several trials between −20 and −45 °C (mimicking low winter temperatures in subalpine Rocky Mountain communities), and found −34 °C to inflict modest but not catastrophic freezing damage ($F_v/F_m \sim 0.400–0.600$). The target temperature was maintained for one hour, and then the temperature was raised at a rate of 1.5 °C h^{-1} and maintained at 3 °C for 48 h post-freezing to accumulate damage. Temperatures were monitored using iButton temperature loggers (Maxim Integrated, San Jose, CA). Freezing damage for the treatment samples was then assessed by measuring dark-adapted F_v/F_m at room temperature with a modulated chlorophyll fluorometer (as in Bykova and Sage, 2012; L'Hirondelle and Binder, 2005; Rizza et al., 2001); F_v/F_m of the control samples were measured concurrently. Cold hardiness was assessed for 1020 seedlings in 2012 and 680 seedlings in 2013, equally distributed among each of the R and S families.

In an additional experiment, we assessed the post-drought cold hardiness of droughted and non-droughted plants from the drought response experiment described previously. These plants were sampled during January, 2014 and subjected to cold hardiness testing (using a Tenney programmable freezer, as described above) to assess the effect of summer drought on subsequent winter cold hardiness. Only plants that had recovered healthy F_v/F_m values (>0.750) after the experience of drought and subsequent re-watering were included in this analysis; of the 180 plants in the drought study, 176 recovered healthy values: 87 from R families and 89 from S families.

In another experiment, we assessed the post-inoculation cold hardiness of inoculated and uninoculated plants the winter immediately following exposure to *C. ribicola*. For this test, we selected 310 plants from the common garden; each family was represented

by 4–9 individuals selected randomly from the common garden for a total of 155 seedlings inoculated with *C. ribicola* and 155 serving as uninoculated controls. Measurements of cold hardiness of these seedlings were conducted in January, 2014 in the same fashion as described previously. The controlled inoculation with *C. ribicola* was conducted at DGRC by the Center's staff during the week of September 12, 2013 following established protocols (Samman, 1982). *C. ribicola*-infected *Ribes hudsonianum* var. *petiolare* leaves from four eastern Oregon areas on the Wallowa-Whitman and Umatilla National Forests near whitebark pine populations were used in the inoculation. *C. ribicola* in eastern Oregon is considered a wild type because it has little or no known virulence to *Cr2* in western white pine or *Cr1* in sugar pine (Kinloch et al., 2003). Spore density averaged 6690 spores cm^{-2}, with an average basidiospore germination (assessed on agar plates) of 98.0%. Seedlings remained in the inoculation chamber for 2 days (20 °C, 100%RH) following the removal of the *Ribes* leaves and were then moved outside alongside the uninoculated controls.

2.4. Data analysis

To assess the comparative cold and drought responses of R and S families of limber pine, we paired an R and an S family from each site of origin ("Site") to filter out any possible geographic variation. As such, we used paired t-tests to evaluate potential R–S differences in the parameters of interest using SAS statistical software (SAS Institute, Cary, NC).

To quantify cold hardiness, we calculated the ratio of F_v/F_m of frozen fascicles to unfrozen controls from the same plant (Pukacki and Kaminska-Rozek, 2005), referred to hereafter as "Freeze: Control F_v/F_m ratio." To ascertain if plants showed similar cold hardiness during the two measurement years, we used a mixed model to determine if an individual's 2012 and 2013 Freeze: Control F_v/F_m ratios were correlated, with Site as a random effect.

For drought response tests, evaluations of potential R and S differences were conducted while plants were experiencing similar levels of drought, specifically "severe" and "modest" drought. In several temperate-zone tree species, drought is considered "severe" at or below plant water potentials of *c.* −2 MPa (Eastman and Camm, 1995; Epron, 1997; Lamy et al., 2014). Correspondingly, we also observed damage, as measured by a decline in F_v/F_m, when seedlings approached or exceeded this value of Ψ_w (Fig. S1) and negative or near-zero net CO_2 assimilation rates (A_{net}). Because water potential was measured using a subset of plants due to time constraints, we also used pot weights in evaluating the degree of water stress across all plants. Generally, plants dropped below −2 MPa after losing ⩾40% of their pot weights (Fig. S2). A loss of 40% or more of pot weight also corresponded to a drop in F_v/F_m (Fig. S3A), usually after plants had been at or above 40% weight loss for 20 days or longer (Fig. S3B). As such, when evaluating the performance of plants under severe drought, we only included seedlings whose pot weight loss exceeded 40% and had done so for at least 20 days. "Modest" drought was defined as a Ψ_w between −1 and −2 MPa and we measured seedling performance during the time frame that average water potential on measured plants was between −1 and −2 MPa and included seedlings with ⩾40% of pot weight loss for 1–20 days. When quantifying plant damage under drought with chlorophyll fluorescence, we used the ratio of F_v/F_m of droughted seedlings with the mean family F_v/F_m for non-droughted controls (hereafter "Drought: Control F_v/F_m ratio"). We also quantified drought response using gas exchange, and individual values of A_{net} in drought plants were divided by the family's mean value of A_{net} for non-droughted plants; this value is referred to as the "Drought: Control A_{net} ratio." A similar calculation was used for evaluating stomatal conductance (g_s).

3. Results

3.1. Drought response

Withholding water markedly impacted soil and plant water statuses and plant activity. Average fascicle pre-dawn water potentials (Ψ_w) dropped below levels for modest drought (*c.* −1.0 MPa) around Day 24 of the drought treatment while non-droughted controls exhibited a mean Ψ_w of −0.16 MPa at this time (Fig. 2A). Correspondingly, mean weight loss from pots was *c.* 40% on Day 24 (Fig. 2C) and mean soil volumetric water content for drought plants was 4.4% compared to 17.0% for non-droughted control plants. Average pre-dawn water potentials exhibited severe plant water stress (*c.* −2 MPa) around Day 46 when mean control Ψ_w was −0.18 MPa.

Gas exchange also reflected the drawdown in soil water content as average net CO_2 assimilation rates (A_{net}) declined by 60.4% (Fig. 2B) and stomatal conductances by 63.6% (Fig. 2D) under modest drought (Day 25). Under severe drought (Day 47), mean A_{net} had fallen to −0.16 μmol CO_2 m^{-2} s^{-1} in drought plants and stomata had mostly closed with g_s declining from 0.026 to 0.006 mol H_2O m^{-2} s^{-1} from Day 1 to Day 47. It is notable that gas exchange in well-watered plants also declined after Day 25 with a drop in A_{net} of 48.5% between Days 25 and 47 (Fig. 2C), potentially due to a seasonal down-regulation of photosynthetic activity during the late summer. This further justifies our use of Drought: Control ratios in this experiment since a decline in absolute A_{net} or g_s in drought plants could have been due to seasonal variation rather than the effects of the drought treatment. Substantial damage to photosystems became apparent generally after Day 56 without water (Fig. S3A); by Day 65, mean F_v/F_m ± standard error (SE) of drought plants was 0.675 ± 0.018 compared to 0.811 ± 0.005 in non-droughted controls.

The physiological response to modest drought of R and S families of limber pine differed. When plants experienced modest drought (Day 25 without water), R families exhibited a significantly lower Drought: Control g_s ratio (paired *t*-test: DF = 9, $t = 2.61$, $P = 0.028$) (Table 3), indicating that R families experienced greater reduction in stomatal conductance than S families, and therefore a greater stomatal limitation to water loss, during modest water stress. The Drought: Control A_{net} ratio was also less for R families under modest drought (Table 3), though not significantly so (paired *t*-test: DF = 9, $t = 1.80$, $P = 0.11$). Under severe drought, R and S families' Drought: Control A_{net} and g_s ratios were not significantly different using a paired *t*-test (DF = 9, $t = 0.79$, $P = 0.45$; DF = 9, $t = 0.12$, $P = 0.91$, respectively) (Table 3), likely a result of near-total stomatal closure among all droughted plants by this time (Fig. 2D). Under both modest and severe drought, damage (as measured by the mean Drought: Control F_v/F_m ratios) was not significantly different for R and S families using a paired *t*-test on family means (modest drought: DF = 9, $t = 0.47$, $P = 0.65$; severe drought: DF = 9, $t = 1.00$, $P = 0.34$) (Table 3).

3.2. Cold hardiness

In general, there was greater cold hardiness in R than S families of limber pine. In autumn, 2012, R families' cold hardiness was significantly greater than S families' (paired *t*-test: DF = 9, $t = 2.34$, $P = 0.044$) with R families exhibiting a Freeze: Control F_v/F_m ratio ± SE of 0.668 ± 0.008 and S families a value of 0.611 ± 0.011 (Fig. 3). In autumn, 2013, R families again showed greater cold hardiness, but not significantly so (paired *t*-test: DF = 9, $t = 1.61$, $P = 0.14$), with R families exhibiting a mean Freeze: Control F_v/F_m ratio ± SE of 0.719 ± 0.007 and S families a value of 0.675 ± 0.009 (Fig. 3). There was a highly significant relationship between

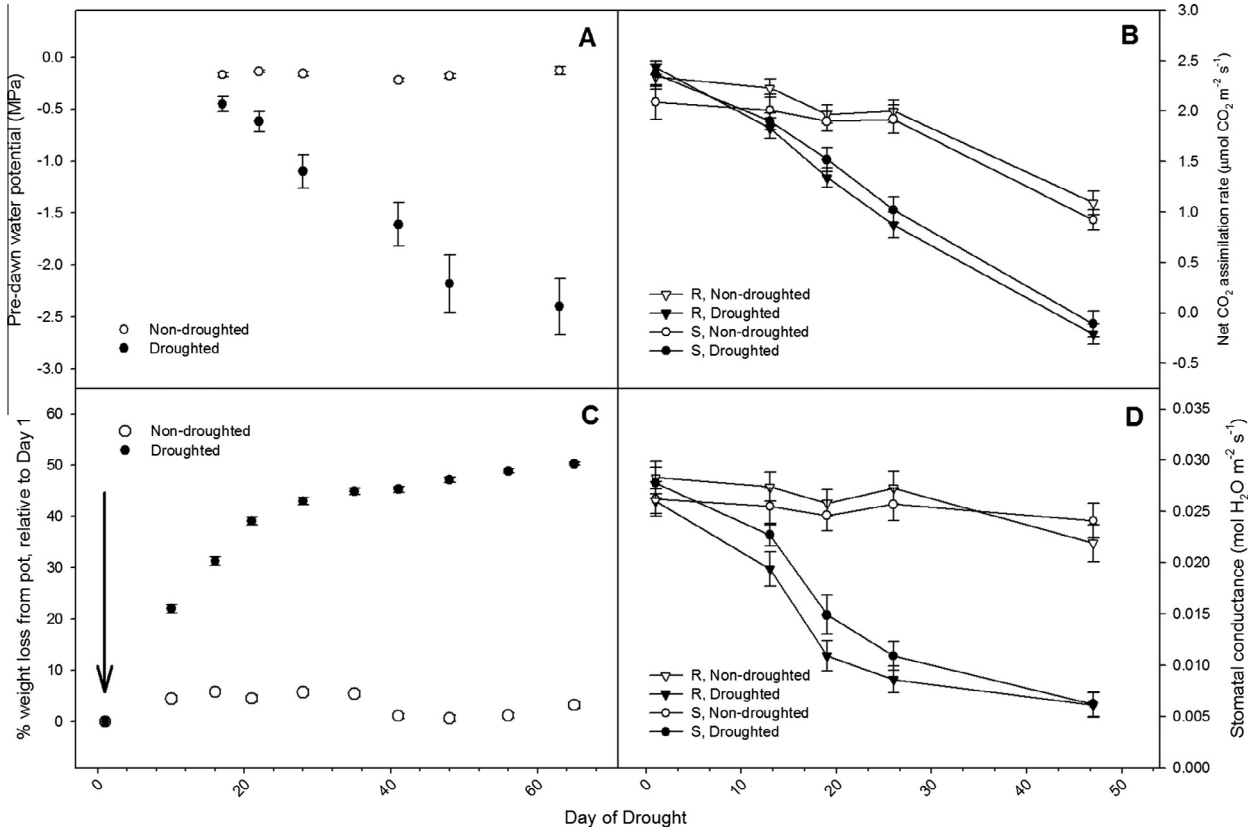

Fig. 2. Since Day 1 of water withholding for seedlings of limber pine (indicated by arrow), the responses over time of (A) fascicle pre-dawn water potentials; (B) net CO_2 assimilation rate; (C) percent weight loss from pots; and (D) stomatal conductance among droughted and non-droughted treatments and between R and S families. Error bars indicate standard error.

Table 3
The drought response of R and S families of limber pine under modest (Day 25–26 of water withholding) and severe (Day 65) drought, expressed as the ratio of the trait for droughted seedlings to non-droughted controls from the same family (±standard errors). Traits include stomatal conductance (g_s), net CO_2 assimilation rate (A_{net}), and chlorophyll fluorescence (F_v/F_m). *P*-values represent the results of paired *t*-tests comparing family averages of paired R and S families from the same site.

Drought	Trait	R family Drought: Control ratio	S family Drought: Control ratio	*P*
Modest	g_s	0.235 ± 0.040	0.439 ± 0.062	0.028
	A_{net}	0.367 ± 0.059	0.585 ± 0.074	0.110
	F_v/F_m	0.990 ± 0.005	0.986 ± 0.008	0.650
Severe	g_s	0.282 ± 0.051	0.275 ± 0.062	0.910
	A_{net}	−0.245 ± 0.106	−0.085 ± 0.155	0.447
	F_v/F_m	0.819 ± 0.031	0.785 ± 0.035	0.340

individuals' 2012 and 2013 Freeze: Control F_v/F_m ratios (mixed model, with Site as a random effect: $F_{(1,5)} = 25.7$, $P = 0.003$), indicating that the trend of greater cold hardiness in R families was broadly conserved between the two measurement times (Fig. S4). Because the 2012 measurements were based on a larger sample size than 2013 (1020 v. 680 seedlings, the latter number being the result of seedling removal for drought and inoculation testing), they provide a more accurate depiction of this relationship, and we thus conclude that these combined datasets support the hypothesis of greater cold tolerance in R families of limber pine.

During January, 2014, we also tested whether inoculation with blister rust during the previous fall induced pathogen-related responses that enhanced peak plant cold hardiness. We found no significant interaction between family resistance status and inoculation, nor did we detect simple effects of inoculation on cold hardiness. Inoculated and uninoculated plants of the same family did not differ significantly in their Freeze: Control F_v/F_m ratios using a paired *t*-test (DF = 19, $t = 1.31$, $P = 0.20$) and showed Freeze: Control F_v/F_m ratios ± SE of 0.911 ± 0.007 and 0.898 ± 0.007, respectively.

3.3. Relationships between drought and cold tolerance

Because some common mechanisms exist in plant cells that enhance both drought and cold tolerance (Seki et al., 2003), we hypothesized that plants exhibiting higher drought tolerances would also have greater cold hardiness. The results of stress tolerance studies indicated no such relationship among limber pine seedlings (Fig. 4). We used a mixed model (with Site as a random effect) to ascertain if a significant relationship existed between damage caused by drought (Drought: Control F_v/F_m ratio) and damage caused by low temperature (Freeze: Control F_v/F_m ratio) of the

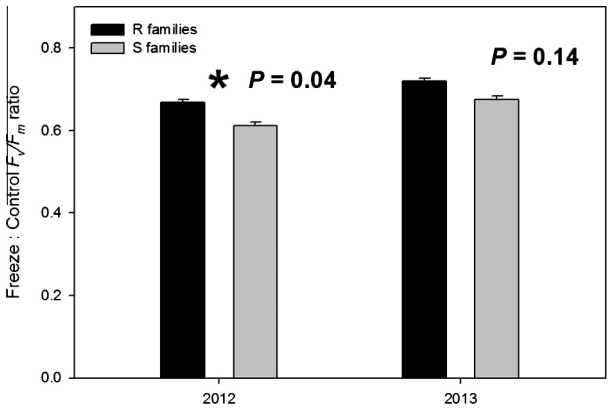

Fig. 3. The cold hardiness of R and S families of limber pine during November, 2012 and 2013, expressed as the ratio of F_v/F_m of frozen fascicles to unfrozen controls from the same plant. Asterisk indicates a significant difference at $P < 0.05$ using a paired *t*-test comparing family averages of paired R and S families from the same site. Error bars indicate standard error.

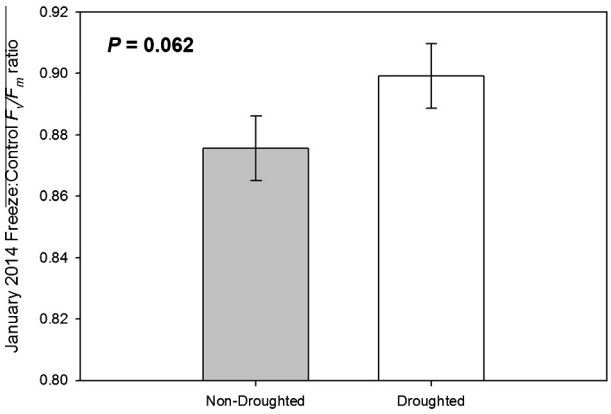

Fig. 5. The cold hardiness in January, 2014 of limber pine seedlings that did and did not experience drought during summer and fall, 2013, expressed as the ratio of F_v/F_m of frozen fascicles to unfrozen controls from the same plant. *P*-value represents the results of a paired *t*-test comparing family averages of droughted and non-droughted plants. Error bars indicate standard error.

autumn, 2012 and 2013 cold tolerance tests. There was no significant relationship between drought tolerance and 2012 ($F_{(1,5)} = 1.32$, $P = 0.30$) or 2013 ($F_{(1,5)} = 1.59$, $P = 0.26$) cold tolerances.

We also hypothesized that the experience of drought during the summer and fall of 2013 may have induced cellular mechanisms that enhanced the cold hardiness of these plants the following winter. When tested for cold hardiness in January, 2014, plants that had experienced drought had a Freeze: Control F_v/F_m ratio ± SE of 0.899 ± 0.011 and non-droughted controls had a value of 0.876 ± 0.011 (Fig. 5). This difference was marginally significant (paired *t*-test: DF = 19, $t = 1.89$, $P = 0.062$), which modestly supports the notion that the experience of summer drought and recovery may have pre-conditioned limber pine seedlings to have greater cold hardiness the following winter.

4. Discussion

Limber pine mortality is increasing across the West as a result of the combined stresses of WPBR, mountain pine beetle, and dwarf mistletoe in a changing climate (Krist et al., 2014). With the continued spread of WPBR, extensive mortality will continue with strong selection against trees that lack genetic resistance to the disease. Genetic resistance to the non-native disease is naturally present in limber pine but at low frequencies raising concern that the WPBR-caused demographic constriction of limber pine populations could also be a genetic bottleneck causing the suite of adaptive stress tolerance traits in the post-selection populations to differ from those of the naïve populations. Here we report that cold hardiness and drought response, two key traits for a species that grows in dry rocky habitats across a range of elevations, differ for WPBR resistant families compared to those that are susceptible, suggesting that WPBR selection is not neutral and can be expected to potentially shift limber pine's performance, especially in a changing climate.

We found significant differences between the performance of seedling families from known rust-resistant (R) and rust-susceptible (S) limber pine seed trees under drought and freezing conditions. Our results reveal that R families exhibited lower stomatal conductance during modest water stress than S families. This indicates that as the frequency of rust-resistant individuals increases in a population with WPBR resistance selection, the resulting populations may be more likely to restrict water loss during mild droughts, which may in turn reduce carbon gain, growth and competitive ability. As there was no significant difference in damage (as measured by Drought: Control F_v/F_m ratio) between R and S families under modest drought or subsequent severe drought, it

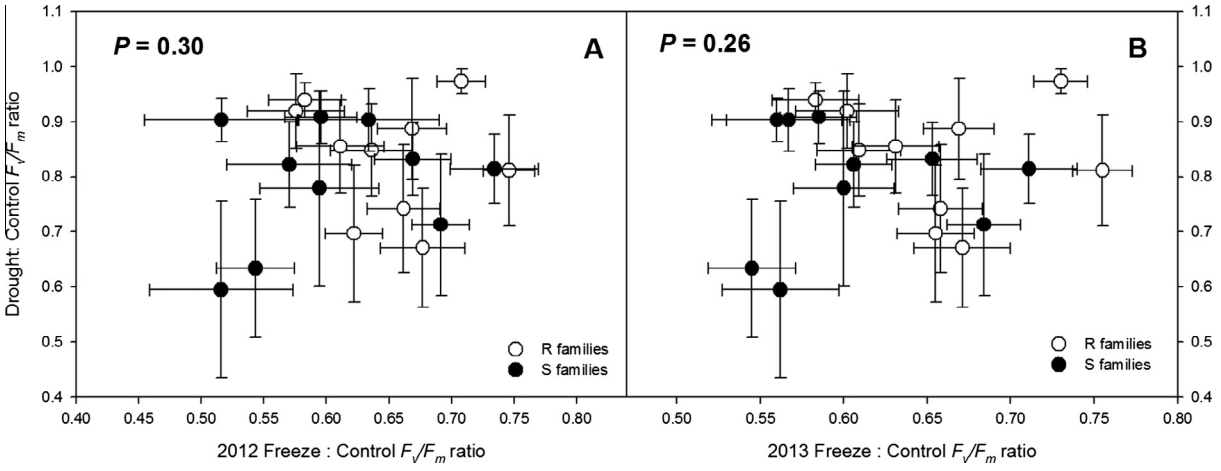

Fig. 4. The relationship between drought tolerance and cold tolerance in R and S limber pine families. Drought tolerance is expressed as the ratio of F_v/F_m of droughted seedlings to non-droughted controls from the same family and cold tolerance is expressed as the ratio of F_v/F_m of frozen fascicles to unfrozen controls from the same plant in (A) November, 2012 and (B) November, 2013. Error bars indicate standard error. *P*-values represent the output of a mixed model with Site included as a random factor.

appears that, in our study, the less conservative strategy of S families did not result in increased damage while plants retained greater rates of leaf gas exchange. Whether the response of R families may confer adaptive advantage in the field over a longer term is complex and uncertain. Limber pine tends to exhibit a conservative strategy of growth, stomatal behavior and resource-use (Pataki et al., 2000; Letts et al., 2009) as well as substantial drought sensitivity across a range of elevations and vegetation cover (Moyes et al., 2013), so this earlier stomatal closure may be viewed as beneficial to R families. Likewise, this difference in leaf conductance under modest drought may have varying impacts in warmer, drier future climates, as more conservative water-use could enhance R families' survivorship and seedling establishment in stressful habitats such as exposed, windswept ridges (Moyes et al., 2013). However, reduced leaf gas exchange may reduce growth and impair competitive ability on more mesic sites or elsewhere if warmer temperatures lead to upslope encroachment of more competitive species and increasing stand density (Letts et al., 2009). Nonetheless, under repeated severe drought episodes we suspect that mortality events such as that reported in limber pine in the Sierra Nevada during a series of dry years from 1985 to 1995 (Millar et al., 2007) will continue and be unaffected by WPBR selection.

R families may be anatomically inclined to maintain lower stomatal conductance through smaller stomatal pore size. It has been noted that individuals of western white pine expressing reduced needle lesion frequency resistance to WPBR have shorter, narrower stomatal pores than rust-susceptible individuals (Woo et al., 2001, 2004) and this may result in a difference in stomatal conductance as well as a smaller entry point for fungal hyphae (Woo et al., 2002). Stomatal density is variable in limber pine across habitats (Schoettle and Rochelle, 2000) indicating that further research on differences in stomatal size and density between R and S limber pine families could be fruitful. Our results support the hypothesis of differential drought response of R and S families. There was no significant difference in drought-induced damage between R and S families, but differences in stomatal conductance under modest drought suggest altered tolerance to stressful habitats and potentially lower competitiveness in post-invasion subalpine forests under a changing climate.

As hypothesized, significantly greater cold tolerance of R compared to S families was observed after cold hardening. Because inoculation with *C. ribicola* did not differentially affect the cold tolerance of R and S families, we conclude that the observed difference in cold tolerance between R and S families is constitutive rather than induced by pathogen infection. Therefore WPBR selection may increase the hardiness of the species to increasing climate variability and extreme cold events. The absence of any difference in mid-winter cold hardiness following exposure to *C. ribicola* suggests that the mechanisms induced by infection in resistant individuals (such as pathogenesis-related proteins) were insufficient in limber pine to alter winter cold tolerance; greater variation may have been detected if measured during the hardening period. Alternatively, it is possible that such mechanisms are operating, but their potential for reducing freezing damage is opposed and effectively nullified by the engagement of a hypersensitive response in individuals with the *Cr4* alelle in R families. Mutations causing continuous engagement of some *R* genes in *Arabidopsis* result in greater sensitivity to cold, possibly due to stress from accumulation of reactive oxygen species as part of the hypersensitive response (Huang et al., 2010).

The correlation of qualitative resistance with greater cold hardiness in limber pine may reflect a close physical relationship between *Cr4* and a gene or genes coding for cold tolerance mechanisms, and there is some evidence from previous assessments of white pines that such a relationship may exist. There is a tendency for *Cr1* in sugar pine to occur with much greater frequency above *c.* 1700 m in the Sierra Nevada and these provenances displayed substantial cold hardiness in reciprocal transplants, though differences in climate along the latitudinal gradient of the Sierra make it difficult to draw conclusions about whether the distribution of *Cr1* is driven by temperature (Kinloch, 1992). The highest frequency of *Cr2* in western white pine is likewise in the high elevations of the southern Sierra Nevada (Kinloch et al., 2003). Many factors including genetic background and isolation as well as local adaptation result in geographic variation in traits; however, because traits for cold tolerance are under strong selection in forest trees (Howe et al., 2003), the distribution of the other *Cr* genes is consistent with the possibility that *Cr4* in limber pine is closely associated with a gene or genes coding for cold hardiness traits. Thus, natural selection for cold hardiness may have contributed to the greater frequency of a *Cr* allele than would be expected from neutral mutation, which may in turn contribute to an explanation of its frequency in naïve populations to a disease that is a recent introduction.

Mahalovich et al. (2006) report a contrary trend for whitebark pine (*P. albicaulis*) where the cline for quantitative resistance to WPBR runs counter to that for cold hardiness within the interior west. These opposing patterns observed here and by Mahalovich et al. (2006) may be due to differences between quantitative and qualitative WPBR resistance mechanisms or may result from the different sampling strategies used. Because rust resistance in the 2006 study (Mahalovich et al., 2006) was confounded by the strong gradient in environmental conditions and possible differences in genetic background between the populations, the effect of the proteins associated with WPBR resistance and freezing tolerance in whitebark pine may have been masked. The paired sampling of R and S families within populations in our study minimizes the confounding effects of environmental gradients. In addition, our sampled populations' likely share the same genetic background as mtDNA analyses suggests that the Northern Colorado and Southern Wyoming limber pine populations are likely products of colonization after the last glacial maximum from the same refugia to the east (Mitton et al., 2000) which may have carried the R allele. Other hypotheses, such as the one proposed by Kinloch (1992), suggest that *Cr1* in sugar pine may have evolved as an adaptation to a biotic stressor – exposure to pinyon rust (*Cronartium occidentale*) – rather than abiotic stressors yet this hypothesis is confounded with geography. Comprehensive testing for qualitative and quantitative resistance across the full range of limber pine will provide further opportunity to evaluate correlations of *Cr4* and polygenic resistance frequencies with climatic variables and past and present biotic interactions.

Another possible explanation for the differing cold tolerances of R and S families is that the protein product of the *R* gene in limber pine directly serves as a cryoprotectant. Many *R* genes, likely including those of sugar pine and western white pine, code for proteins with leucine-rich repeats (LRRs) (Jermstad et al., 2006; Liu and Ekramoddoullah, 2008), a motif that is key to disease resistance due to its capacity for rapid evolution (Bergelson et al., 2001). In addition to their important role in disease resistance, leucine-rich repeats also have demonstrated ice-binding or antifreeze capability (Worrall et al., 1998; Zhang et al., 2004; Griffith and Yaish, 2004; Muthukumaran et al., 2011). Further research is needed to explore the association between *Cr* and leucine-rich repeat-containing proteins and their role in cold hardiness.

Our results do not support a positive correlation between cold and drought tolerances, as measured by damage to photosynthetic machinery, in limber pine. However, there is some evidence ($P = 0.062$) that the experience of drought and recovery may enhanced subsequent winter cold hardiness. Our results add to the mixed results of prior research in conifer seedlings where drought hardening increased (Timmis and Tanaka, 1976; Blake

et al., 1979) or reduced (Grossnickle et al., 1991) or had no effect (Arnott et al., 1993) on subsequent cold hardiness.

The correlations between *Cr4* and drought response and cold hardiness traits we report here are conservative. In open-pollinated families approximately 50% of the progeny from a *Cr4cr4* seed tree will inherit the *Cr4* allele and the rest are rust-susceptible. Because not all individuals in each R family possess the *Cr4* allele, the impact of resistant individuals in R families is diluted in the observed trends. Determining the phenotype, and therefore the genotype, of each seedling through subsequent inoculation with *C. ribicola* and reanalysis of the data to compare traits of seedlings confirmed to have *Cr4* or not may improve the strength of the reported correlations. Likewise, our results on cold hardiness are based on analyses of needles only, not whole plants; yet, the leaves are the infection- and, suspected to be, the host-response court for qualitative WPBR resistance. Our results suggest a possible physiological link between disease resistance and stress tolerance, yet inferences about overall plant success in different environments should be made cautiously.

We conclude that as the frequency of qualitative resistance to WPBR increases in populations, through natural selection in the field or artificial selection by management, the resultant populations may have a different suite of stress tolerance traits than pre-invasion populations. As hypothesized, we observed a correlation between qualitative resistance to WPBR and enhanced cold hardiness in limber pine, and a differential response to modest drought between R and S families. While resistance to WPBR is the most important adaptive trait determining the sustainability of limber pine populations into the future, the loss of traits correlated with WPBR susceptibility resulting in a possible shift in the fundamental niche of the species is important to identify and target suitable habitat for R genotypes and select appropriate seed sources for successful restoration.

This research highlights how plant adaptations to biotic and abiotic stresses are intertwined and understanding these relationships can inform successful management of natural populations challenged by non-native pests and pathogens. The strong directional selection pressures on native populations imposed by introduced pests and pathogens, or management to mitigate them, may have lasting genetic effects on species that need to be considered when planning restoration strategies in a changing climate.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foreco.2015.01.029.

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