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Breeding for cold tolerance in common annual legume cover crops

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Abstract

There has been a significant increase in cover crop adoption, with land use nearly doubling over the last decade. Winter legume cover crops provide several ecosystem services, such as weed suppression and reducing soil erosion, while serving as an excellent nitrogen source for subsequent cash crops. Hairy vetch (*Vicia villosa* R.), crimson clover (*Trifolium incarnatum*), and winter pea (*Pisum sativum* L.) are three major winter annual legume cover crops in the United States. However, varying winter survival rates have reduced their reliability compared with winter hardy grasses like cereal rye (*Secale cereale*). Winter hardy cultivars have been selected and bred in winter pea, which are also used as food and forage crops, but fewer breeding efforts toward cold tolerance have been made in hairy vetch or crimson clover. Despite the current breeding efforts, all three species can suffer from winter damage in the winter hardiness zone 6 and below. Developing winter hardy annual legume cover crops requires a multifaceted approach, including cultivar selection and hybridization, quantitative trait locus isolation, management practice improvement, and identification of new sources of winter hardiness. Cold acclimation, deacclimation resistance, and reacclimation potential are possible mechanisms to explore in the cold tolerance of these winter cover crops. Cold tolerance can be evaluated in field and controlled environments using visual scoring, chlorophyll fluorescence, and ion leakage assays.

Plain Language Summary

Winter legume cover crops are plants grown to reduce weeds, prevent soil erosion, and add nitrogen to the soil for use by subsequent crops. Some common winter legume cover crops in the United States are hairy vetch, crimson clover, and winter pea. However, these plants do not consistently survive the winter, which limits their benefits and hinders their adoption. While there have been efforts to improve cold tolerance in winter pea, efforts have been more limited in hairy vetch and crimson clover.

Abbreviations: CBF, C-repeat binding factor; CCB, cover crop breeding; COR, cold-regulated gene; GS, genomic selection; LEA, late embryogenesis abundant; LT50, lethal temperature at which 50% of plants die; QTL, quantitative trait locus; ROS, reactive oxygen species; VNS, variety not stated.

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Despite the current breeding efforts, all three species suffer from winter damage in the northern United States. Efforts such as cultivar selection and breeding, identifying genetic markers, improving management practices, studying cold physiology, and identifying new sources of winter hardiness are needed to develop winter hardy cover legume cover crops. Winter hardy cover crops grow more vigorously, control weeds better, and offer more environmental benefits than winter killed cover crops.

1 | INTRODUCTION

A cover crop is a plant that is grown to benefit the soil and/or other crops rather than for harvest (though cover crops are sometimes also harvested or grazed). Cover crops are often planted in periods between primary cash crops, eliminating fallow periods and providing numerous benefits to the soil, including protection from erosion (Baets et al., 2011; Seo et al., 2005), improved infiltration (Haruna et al., 2022; Rauber et al., 2025), increased water-holding capacity (Basche et al., 2016; Irmak et al., 2018), and increased organic matter (Vieira et al., 2009). Cover crops can also be used to manage weeds (Baraibar et al., 2018; Hiltbrunner et al., 2007), pests (Beaumelle et al., 2021; Tillman et al., 2004), and diseases (Panth et al., 2020; Wen et al., 2017).

The benefits obtained from cover crops vary by family and species used (Kladivko, 2016; Snapp et al., 2005). For instance, legume cover crops fix nitrogen (N), providing fertility for subsequent crops, while grasses and brassicas scavenge residual N to reduce leaching (Dabney et al., 2001). Among legumes, hairy vetch is the most winter hardy, while crimson clover accumulates more aboveground N and matures earlier than hairy vetch and winter pea (Gatiboni & Reberg-Horton, 2021). Crimson clover and hairy vetch are more effective at weed suppression compared to winter pea (Vann et al., 2019). Hairy vetch produces hard seed and can become a weed in the subsequent crops (Maul et al., 2011; Vann et al., 2019), but efforts are underway to ameliorate this problem (Tilhou et al., 2023, 2024). Introducing a legume cover crop into the winter cover crop rotation provides several ecosystem service benefits. It can decrease the N requirement, and thereby the fertilizer cost, of the following cash crop (Foote et al., 2014). For example, winter pea can fix up to 168 kg/ha N, which becomes available to subsequent crops (Clark, 2007). Furthermore, planting a legume cover crop rather than rye before corn or another cash crop in the Poaceae family can mitigate the risk of transferring pests and pathogens from rye to the cash crop (Dawadi et al., 2019; Hart, 2020; Snapp et al., 2005). Alternating crop families can remove the preferred habitat and food sources for pests adapted to specific plant types and break the life cycle of many pathogens (Mohler & Johnson, 2009). However, the northern United States (USDA plant hardiness zone 6 and below) has very harsh winters, which lead to low and inconsistent winter survival of most cover crops, including winter annual legumes. Winter killed cover crops, compared to winter hardy cover crops, produce less biomass (Florence et al., 2019), suppress weeds less effectively (Ranaldo et al., 2020), lead to higher N leaching (Gollner et al., 2020), and have decreased total ecosystem services due to their shorter lifespan (Kaye et al., 2019; White et al., 2016). Even in winter hardy cover crops, cold stress reduces nodulation and nitrogen fixation (Thurston et al., 2022).

Hairy vetch (*Vicia villosa* R.), crimson clover (*Trifolium incarnatum*), and winter pea (*Pisum sativum* L.) were the most widely planted winter annual legume cover crops in the United States from 2014 to 2020 (SARE-CTIC, 2016, 2017, 2020). Crimson clover and hairy vetch remain the two most widely sown winter annual legume cover crops, but balansa clover (*Trifolium michelianum*) became more widely grown than winter pea starting in 2022 (SARE-CTIC, 2023). Among 575 respondents, crimson clover, hairy vetch, balansa clover, and winter pea were planted in 14,133, 6872, 4991, and 4717 ha, respectively (SARE-CTIC, 2023). Balansa clover has increased in popularity in 2022–2023 compared to 2019–2020 (SARE-CTIC, 2020, 2023).

Although crimson clover is primarily grown in the southern states, it has been successfully introduced as a winter annual cover crop as far north as northern Maine (USDA-NRCS, 2024).

This paper reviews winter hardiness in three commonly grown annual legume cover crops: winter pea, hairy vetch, and crimson clover. We describe their hardiness levels and potential cold hardy mechanisms, as well as current breeding efforts, techniques, and progress in improving winter hardiness in these crops. Finally, we discuss prospects for developing winter hardy annual legume cover crops in the northern US regions.

2 | CURRENT LEVEL OF WINTER HARDINESS IN COMMON ANNUAL WINTER LEGUME COVER CROPS

2.1 | Hairy vetch

Hairy vetch, though considered the most winter hardy annual legume cover crop, has inconsistent winter survival across years and geographical locations in the northern United

States. Weather parameters, such as ice sheeting, lack of snow cover, and low-temperature periods without snow cover, reduce the winter survival of current cultivars (Kucek et al., 2024).

Variety not stated (VNS) seed is widely used for cover cropping since it is typically cheaper and more widely available than named cover crop cultivars. However, VNS seed generally lacks information about pedigree, specific characteristics, or regional performance (Reed et al., 2024), leading to unpredictable outcomes in nontarget environments. For outcrossing species grown as open-pollinated populations, the seed production environment has an influence on traits including winter hardiness, so VNS seed may have desirable characteristics if seed is produced in an environment similar to the target environment. For example, Moore et al. (2020a) found that a Nebraska-grown VNS hairy vetch population had superior winter hardiness. But information about the seed production environment is often not available, and in the absence of this information, specific characteristics will be unpredictable among locations and years. Efforts to select hairy vetch for winter survival (and related traits) have been initiated (Table 1) but have so far been limited. Winter survival of hairy vetch accessions ranged from 13% to 73% across three locations in Minnesota (Wiering et al., 2018) and from 55% to 99% across 15 US locations (Kucek et al., 2024). Harbur et al. (2009) found that Minnesota ecotypes had a winter survival rate of 40%–60%, significantly higher than non-Minnesota ecotypes with survival rates of 14%–36% in the same environment. Significant annual variation in winter conditions leads to unpredictable outcomes for winter survival in nurseries and trials. This highlights the value of multilocation selection and evaluation with strategic placement of breeding, trialing, and seed production sites. A national cover crop breeding (CCB) network, including collaborators at USDA Agricultural Research Service (ARS) and multiple universities, has initiated breeding programs to improve winter hardiness of legume cover crops, including hairy vetch (Kucek et al., 2024). Expanding similar efforts and identifying materials with consistently high winter survival could accelerate the breeding process for cold tolerant cultivars.

2.2 | Winter pea

Winter damage is a significant factor limiting the adoption of winter pea as a cover crop and food crop in the northern United States (Vann et al., 2019). Efforts to screen for winter hardiness in pea dates back to the 1960s (Dowker, 1969). Controlled environment and field experiments have identified pea germplasm that can survive at low temperatures of −9°C to as low as −15°C, exhibiting minimal to no visible damage and achieving up to 80% winter survival (Auld et al., 1983; Homer et al., 2019; Liu et al., 2017; Zhang et al., 2016) (Tables 1 and 2). However, current commercial cultivars do not consistently survive below −12°C (Richard & Allison, 2020; Vann et al., 2021). Previous germplasm screenings (Liu et al., 2017; Zhang et al., 2016) can serve as valuable sources of information to identify winter hardy germplasm for future cultivar development. Limited screening and inconsistent winter survival (Tables 1 and 2) highlight the need for further efforts to improve winter hardiness in peas.

Core Ideas

- Winter annual legume cover crops show inconsistent survival in USDA plant hardiness zone 6 and below.
- Breeding efforts on cold tolerance focus on winter pea, with limited work on other legumes.
- Possible cold tolerance mechanisms include acclimation, deacclimation resistance, and reacclimation potential.
- Visual scoring, chlorophyll fluorescence, and ion leakage are common methods to assess cold tolerance.
- Breeding, genetic, and management studies related to cold tolerance are needed to improve winter hardiness.

2.3 | Crimson clover

Crimson clover has been grown as a forage or cover crop in the southeastern United States since the 1800s (Hollowell, 1951). Despite its long history of cultivation in southern states, research on the winter survival and adaptability of crimson clover in northern regions has been relatively limited. Crimson clover cultivar Heusers Ostsaat demonstrated 70%–86% winter survival in field and controlled environments (Brandsæter et al., 2000, 2008) (Tables 1 and 2).

Current crimson clover cultivars and accessions exhibited 52%–82% winter survival in Betsville, MD, but less than 50% survival in Big Flats, NY, and Alderson, WV (Moore et al., 2020a; USDA-NRCS, 2020). High winter survival in locations with mild winters reflects crimson clover's development for areas with moderate winter temperatures (Vann et al., 2019). Considering the limited research on crimson clover and its status as the most commonly planted legume cover crop, further screening and selection studies are needed to uncover the cold tolerance potential of this legume.

3 | COLD DAMAGE MECHANISMS

Cold damage in plants can be caused by low temperatures that are above freezing (i.e., chilling, which occurs between 0 and

TABLE 1 Winter survival of current cultivars of hairy vetch, winter pea, and crimson clover in field.

Species	Winter hardy cultivars	Testing environments	Percentage survival	References
Hairy vetch	Groff	Elsberry, MO; Aberdeen, ID	85–87	(Herget, 2020; V. M. Moore et al., 2020a)
	Accession V01 (source: UMN population)	St. Paul, Becker, & Roseau, MN	73	(Wiering et al., 2018)
	Purple bounty	Elsberry, MO; Aberdeen, ID; Pullman, WA	32–84	(Herget, 2020; V. M. Moore et al., 2020a)
	Purple prosperity	Elsberry, MO; Aberdeen, ID; Pullman, WA	16–85	(Herget, 2020; V. M. Moore et al., 2020a)
	Villana	Elsberry, MO	84	(Herget, 2020)
	TNT	Pullman, WA	50	(V. M. Moore et al., 2020a)
Winter pea	Melrose	Ulas, Silas Turkey; Moscow, ID; Bozeman, MT	100	(Auld et al., 1983; Homer et al., 2016)
	PI 392019	Ulas, Silas Turkey	100	(Homer et al., 2016)
	Accession G0002295, PI 269818	Binzhou, Yantai, and Qingdao, China	75	(Liu et al., 2017)
	Lynx	Betsville, MD; Pullman, WA	80	(Casey & Spellman, 2020; Vann et al., 2021)
	Windham	Pulman, WA	73.5	(Casey & Spellman, 2020)
	Frost master	Pulman, WA	63.5	(Casey & Spellman, 2020)
	Fenn	Moscow, ID; Bozeman, MT	72–81.5	(Auld et al., 1983)
	C26-25-26	Moscow, ID; Bozeman, MT	73.5–80	(Auld, et al., 1983)
	Common	Bozeman, MT; Moscow, ID	74.5–82.3	(Auld, et al., 1983)
	Population B1, B6 and B12	Konya, Turkey	100	(Ceyhan, 2010)
Crimson clover	Heusers ostsaat	AS, Norway	70	(Brandsæter et al., 2000)
	Accession PI 556990	Beltsville, MD	82	(V. Moore et al., 2020b)
	Kentucky pride	Ewing, IL; Elsberry, MO; Pullman, WA	44.5–94	(Casey & Spellman, 2020; Demasi, 2019; Herget, 2020)
	Dixie	Elsberry, MO; Pullman, WA	53.5–66	(Herget, 2020) (Casey & Spellman, 2020)
	Contea	Elsberry, MO	63	(Herget, 2020)
	AU Robin	Pullman, WA	49.5	(Casey & Spellman, 2020)
	AU Sunrise	Pullman, WA	45	(Casey & Spellman, 2020)
	AU Sunup	Pullman, WA	39.5	(Casey & Spellman, 2020)

TABLE 2 Percentage survival of winter pea and crimson clover cultivars exposed to −9°C in a controlled environment.

Species	Winter hardy cultivars	Percentage survival	References
Winter pea	Melrose	62%	(Auld et al., 1983)
	Fenn	72%	
	C26-25-26	80%	
Crimson clover	Heusers ostsaat	86.7%	(Brandsæter et al., 2000)

15°C) and below freezing (0°C) (Cooper, 2016). Cold damage is very common during winter in the northern United States. While cold damage physiology has not been studied in hairy vetch and crimson clover, mechanisms of such damage have been well-studied in pea (Dumont et al., 2009, 2011; Klein et al., 2014; Shafiq, 2012). It is hypothesized that the plants perceive low temperatures via yet-to-be-defined sensors, which orchestrate adaptive physiological responses such as a decrease in lipid saturation to maintain cell membrane fluidity (Miura & Furumoto, 2013; Örvar et al., 2000). In chilling-susceptible plants, chilling stress impedes normal metabolic and physiological processes (Lukatkin et al., 2012; Ruelland

et al., 2009). This stress impacts chlorophyll biosynthesis and damages chloroplast structures (Adam & Murthy, 2014). It impedes the synthesis of ATP necessary for the regeneration of ribulose-1,5-bisphosphate to support CO_2 fixation (Ruelland et al., 2009). It also alters chlorophyll antenna complexes and the interaction between different components of photosystem II (Ensminger et al., 2006). As a result, chlorophyll antenna complexes trap excess energy and thylakoid membranes become over energized, resulting in increased reactive oxygen species (ROS) production and photodamage (Ruelland et al., 2009). Moreover, the compromised efficiency of ROS scavenging systems together with the increased reducing power at the acceptor side of photosystem I result in photoinhibition and lipid peroxidation. Thus, chilling leads to lower photosynthesis activity, increased membrane rigidity, loss of electrolytes, and changes in enzyme function and metabolic processes (Lucau-Danila et al., 2012). After a prolonged exposure to chilling, susceptible plants exhibit chilling symptoms such as surface lesions and tissue with a water-soaked appearance (Saltveit & Morris, 1990). Chilling stress is the primary cold stress experienced by plants in tropical regions. However, in temperate and continental regions where freezing temperatures regularly occur, chilling may not cause damage to plants but rather could induce cold acclimation to increase freezing tolerance (Guo et al., 2018). Winter hardy annual legume cover crops undergo acclimation at chilling temperatures but could be susceptible to damage from freezing temperatures.

Freezing injury occurs when ice crystals form in plant tissues (Wang & Wallace, 2004). During freezing stress, intercellular fluid forms ice before intracellular fluid, resulting in a decreased water potential outside of the cell, drawing liquid water from inside the cell to intercellular space (Thomashow, 1999). At −10°C, more than 90% of the osmotically active water typically moves out of the cells to the intercellular spaces (Steponkus & Lynch, 1989), which can cause multiple types of damage to the cell membrane, such as expansion-induced lysis, lamellar-to-hexagonal-II phase transitions, and fracture jump lesions (Uemura & Steponkus, 1997). Various ions, proteins, and lipids are leaked from damaged cells, chlorophyll degrades, and vigor and abscission are lost (Cheng et al., 2010; Steponkus, 1984). Thus, severe dehydration during cold stress can damage the plasma membrane, which is the most serious type of damage (Cheng et al., 2010; Steponkus, 1984).

Brief exposures to low temperatures may only cause transitory changes, while prolonged exposure to cold stress causes necrosis or death (Theocharis et al., 2012). General mechanisms of cold damage are similar across species. However, the level of hardiness and the extent of damage differ among species and genotypes in an environment-dependent manner. Assessing the level of damage in different genotypes, species, and growth locations can inform the impact of low temperature on ecosystem services offered by each legume cover crop and the need for selection and breeding of winter hardy cultivars.

4 | COLD AVOIDANCE AND TOLERANCE

Plants perceive the low temperature signal and transduce it to downstream components that induce appropriate physiological and molecular adaptations and tolerance mechanisms (Theocharis et al., 2012). Resistance to cold stress includes both avoidance and tolerance (Levitt, 1980). Avoidance refers to the prevention of ice formation in its tissues, which can be achieved through freezing point depression, supercooling, and physical barriers to nucleation (Burke et al., 1976; Chen et al., 2015; Clements & Ludlow, 1977; Palta & Li, 1979). Supercooling keeps water in a liquid state at freezing temperatures, which is based on the freezing point of pure water being at approximately −40°C (Ambroise et al., 2020). It is a common phenomenon of cold avoidance in woody plants above the supercooling point (Londo & Kovaleski, 2019). Freezing point depression occurs when certain cryoprotectants delay the formation of ice nucleation (Jahed et al., 2023). Physical barriers to ice nucleation (presence of less hydrophobic surfaces) prevent/delay the formation of ice crystals in woody plants (Gorb & Gorb, 2022). To this date, such avoidance mechanisms have not been studied in pea or other legume cover crops.

On the other hand, cold tolerance refers to a plant's ability to *withstand* chilling and freezing. It is a complex process governed by diverse genetic mechanisms and involves multiple regulatory pathways (Clements & Ludlow, 1977). Chilling tolerance in peas was shown to be associated with expression of C-repeat binding factor (CBF), cold-regulated gene (COR), and late embryogenesis abundant (LEA) genes, and freezing tolerance was associated with cellular osmotic stabilization, photosynthesis modifications, antioxidant production, modifications in hormone metabolism, and cell wall composition and dynamics (Lucau-Danila et al., 2012). Various physiological and biochemical changes, such as changes in protein levels, reduction in metabolism, and increase in antioxidant capacity (Huang & Guo, 2005), improve cold tolerance in plants (Janmohammadi et al., 2015). Winter hardy plants can employ various protective mechanisms against cold stress (Yamazaki et al., 2009). Shared cold avoidance and tolerance mechanisms in annual legumes are described below.

4.1 | Osmoprotectants

Osmoprotectants (sometimes referred to as cryoprotectants) function via both avoidance and tolerance mechanisms, depending on context and concentration. In all plant species,

winter hardy plants tend to have a higher level of osmoprotectants, such as sugars, and a higher osmotic concentration (Dexter, 1956). Plants synthesize osmoprotectants such as glucose, sucrose, raffinose, fructose, suberin, and lignin that help to maintain cellular osmotic balance and protect cellular structures from cold induced damage (Jahed et al., 2023). Osmoprotectants protect cellular membranes from dehydration and freezing damage by interacting with the lipid bilayers (Anchordoguy et al., 1987; Shalaev & Steponkus, 2001). The higher osmotic potential lowers the freezing point of the cell, reduces or resists ice recrystallization in extracellular spaces, and resists desiccation. These constitute avoidance mechanisms of osmoprotectants. Osmoprotectants also operate via tolerance mechanisms, which play crucial roles in protecting cellular structures and functions even after ice forms. Winter hardy cultivars also have a better capability to adjust their photosynthesis apparatus to resist photoinhibition (Lucau-Danila et al., 2012). The effect of osmoprotectants on the cold tolerance of hairy vetch and crimson clover has not been studied. Lucau-Danila et al. (2012) found that genes involved in the synthesis of several amino acids were upregulated in cold tolerant peas. There is a lack of research identifying the specific osmoprotectants that influence the cold tolerance of winter pea.

4.2 | Acclimation

The ability to withstand low temperature conditions in winter hardy legume plants such as peas is directly affected by cold acclimation. Cold acclimation includes chilling tolerance and the induction of freezing tolerance (Bahrman et al., 2019). It is a complex process, driven by a slow transition to low temperatures (Grimaud et al., 2013) and a programmed and integrated genetic capacity to activate mechanisms to withstand freezing conditions. This process results in the expression of CORs, leading to various physiological, morphological, biochemical, and molecular changes. These changes include the accumulation of osmolytes, amino compounds, and soluble sugars, as well as a reduction in general metabolic functions, photosynthesis, protein synthesis, and water and mineral uptake, ultimately increasing cold tolerance (Adam & Murthy, 2014; Bhat et al., 2022; Levitt, 1980; Thomashow, 1990). The genes and mechanisms involved in cold acclimation can vary by species, necessitating detailed studies on each species.

Cold acclimation leads to changes in enzymatic activity, chloroplast thylakoid structure, chlorophyll content, photosynthetic enzyme activity, and electron transport (Adam & Murthy, 2014). Lucau-Danila et al. (2012) found that enzymes involved in cell wall modification and genes involved in synthesis of several amino acids and amines were upregulated in a tolerant cultivar but downregulated in a sensitive cultivar. Similarly, genes associated with photosynthesis were downregulated in a tolerant cultivar and upregulated in sensitive plants. Bahrman et al. (2019) found that two genes, the first being a mediator of the RNA polymerase II transcriptional subunit 32 (MED32, AT1G11760), which regulates the expression of the second, a dehydration element B1A (DREB1A, AT4G25480), both of which have roles in cold acclimation in Arabidopsis, were upregulated in tolerant pea cultivar.

Many genes are induced during cold acclimation. Lucau-Danila et al. (2012) found that the chilling tolerance or sensitivity in pea lines was related to expression responses of the CBF, COR, and LEA genes, with earlier expression of these genes associated with tolerant genotypes. Freezing tolerance was associated with acclimation processes such as cellular osmotic stabilization, photosystem modifications, increased antioxidant production, and modifications in hormone metabolism, cell wall composition, and cell wall dynamics.

The acquisition of cold tolerance through cold acclimation should be considered under specific environmental conditions and in a species-dependent manner (Fiebelkorn, 2013; Gusta & Wisniewski, 2013; Huner et al., 1993). In a study on winter pea, cold tolerance reached its maximum at 4 weeks of acclimation when comparing acclimation periods between 1 and 7 weeks (Swensen & Murray, 1983). Cold tolerance capacity of hairy vetch also increased when the acclimation period was increased from 2 weeks to 4 weeks (Wiering et al., 2018). The effect of acclimation period on cold tolerance of crimson clover is not documented.

4.3 | Deacclimation and reacclimation

Increases in mean temperatures and temperature fluctuations may interfere with the cold acclimation and cold tolerance of winter annual legumes. Consequently, the adaptability of these legumes to cold climates will increasingly depend on their resistance to deacclimation and their ability to reacclimate (Wiering, 2021). Deacclimation is reverse acclimation, or reduction of the level of cold tolerance that was attained through an earlier acclimation process, while reacclimation is the process of regaining some or most of the lost cold tolerance when the deacclimated plant is again exposed to low temperatures (Kalberer et al., 2006). Typically, deacclimation and reacclimation occur in late spring or early summer when temperatures start to rise. However, with more frequent warm spells associated with climate change, these cycles can occur more frequently, even in mid-winter or early spring when the risk of freezing temperatures is significant (Kalberer et al., 2006; Rapacz et al., 2014). Thus, cultivars that are currently well-adapted to a region might not exhibit the same level of adaptation in the future due to increasing temperature fluctuations associated with climate change.

While temperature is the driving force of deacclimation and reacclimation, various environmental and physiological

factors, such as light, carbohydrate reserves and metabolism, drought, flooding, nutrient status, and atmospheric CO_2 concentration, interact with temperature to influence these processes (Rapacz et al., 2014). Furthermore, deacclimation resistance and reacclimation capacity can vary by species, the duration of deacclimation, and the temperature range during deacclimation (Kalberer et al., 2006; Vyse et al., 2019). The capacity for reacclimation becomes more limited as the degree or duration of warm temperature exposure increases and deacclimation advances (Kalberer et al., 2006). Wiering (2021) reported that 1 week of deacclimation at temperatures of 16/6°C (day/night) reduced cold tolerance in both hairy vetch and winter pea genotypes. He also reported that genotypes' interactions with acclimation effects (acclimation, deacclimation, and reacclimation) were significant in winter pea but not in hairy vetch, suggesting that genotype-by-environment interaction may be more important for some species than others. Moreover, greater variability in certain species for acclimation effects might allow more progress in breeding for tolerance to temperature variability. Although some studies have been performed in cereals (Rapacz et al., 2008, 2017), Arabidopsis (Byun et al., 2014), and woody plants (North et al., 2022), knowledge of deacclimation and reacclimation is very limited in winter annual legumes. In environments where deacclimation events are of greater concern, assessing cold tolerance in different scenarios, such as after acclimation and deacclimation, may provide more pertinent information for cold tolerance (Wiering, 2021). Cold tolerance and resistance to deacclimation are independent traits, and even very cold tolerant plants can be susceptible to deacclimation (Wójcik-Jagła & Rapacz, 2023). Thus, studies on deacclimation and reacclimation potential are important for developing winter hardy annual legume cover crops.

5 | BREEDING METHODS FOR COLD TOLERANCE

5.1 | Field evaluation

Field testing is one of the most common approaches for assessing winter hardiness in the context of a breeding program (Wiering et al., 2018). Besides cold tolerance, winter hardiness involves tolerances for ice-encasement, flooding, soil heaving, desiccation, saturated soil, and biotic factors, as well as avoidance of winter conditions through photoperiod sensitivity or pleiotropy (Michel et al., 2019; Murray et al., 1988; Rognli, 2013). Unlike controlled environment studies, which primarily assess cold tolerance, field experiments provide a more comprehensive evaluation of winter hardiness by considering the suite of factors that affect winter survival in natural conditions (Fiebelkorn, 2013). However, the variability in weather conditions each year, along with the irregular occurrence of winter damage in field trials, can hinder phenotypic selection for winter hardiness, making it time-consuming and inconsistent. Field evaluations have been used to screen germplasm for winter hardiness in various crops, including winter pea (Liu et al., 2017), hairy vetch (Wiering et al., 2018), and crimson clover (Brandsæter et al., 2008).

5.2 | Controlled environment and semi-controlled environment evaluation

Controlled environments are commonly used to assess cold tolerance. A controlled environment study can offer invaluable insights into factors that are challenging to observe under field conditions, such as specific effects of low temperature or responses at particular temperature ranges (Langstroff et al., 2022). While controlled environment phenotyping should not be considered a substitute for field phenotyping, it can serve as an initial screening method to assess cold tolerance and identify materials for field testing. A meta-analysis by Poorter et al. (2016) found modest relationships between controlled environment and field trial data. To improve the correlation, several measures can be taken to more closely approximate field conditions in controlled environments, such as mimicking outdoor light and temperature cycles or using larger pots. Homer et al. (2016) also found contrasting results between field and controlled environment evaluations, observing that the survival rate of the same cultivar in a field study at Ulas, TK, and Haymana, TK, with a minimum air temperature of −20.3°C, was higher than the controlled environment study where the lowest temperature was set to −12°C. Several field parameters, such as snow accumulation, may have contributed to improved winter survival observed in the field. Cold tolerance evaluation in controlled environments has previously been conducted in peas (Dumont et al., 2009) and hairy vetch (Wiering et al., 2018) but not in crimson clover.

Semi-controlled environments with snow-out shelters are another proposed approach to assess cold tolerance in the field (Sieber et al., 2014). Snow is hypothesized to protect plants from severe winter injury. A semi-controlled environment could expose plants to other field conditions while preventing snow coverage. For instance, a test named "Weihenstephaner Auswinterungsanlage" proposed by Hoeser (1954) involved growing plants in wooden boxes placed 1 m above the ground. Sieber et al. (2014) tested this method and found a good correlation with field trials. However, this method has not been widely used.

5.3 | Genomic selection

Field testing for winter hardiness is inconsistent, and controlled environment evaluations for cold tolerance are costly,

laborious, and have limited predictive ability. A genomic breeding strategy for early generation selection is a potentially attractive alternative that can increase genetic gain for complex phenotypes like winter hardiness (Jannink, 2010; Michel et al., 2019). Genomic selection (GS) is a technique used to predict traits earlier in the breeding cycle by using genome-wide molecular markers and phenotypic data (Burgueño et al., 2012; Crossa et al., 2010; Heffner et al., 2009). An individual's breeding value is estimated based on genome-wide markers and using a training population consisting of individuals with both genotypic and phenotypic data (Meuwissen et al., 2001). A GS prediction model would be particularly useful for low-temperature injury since weather that causes differential responses may not occur in all environments where evaluation is performed. GS, either for the best-performing lines or against the least winter hardy material, has the potential to increase the efficiency of the selection process compared to relying solely on phenotypic selection (Annicchiarico et al., 2020). However, the prediction accuracy may vary depending on the species and the trait of interest. Annicchiarico et al. (2019) reported that inter-environment predictive ability using intra-population predictions was 0.30 for yield, 0.65 for the onset of flowering, 0.64 for seed weight, and 0.28 for lodging in peas and reduced to 0.19 for grain yield, 0.40 for the onset of flowering, 0.28 for seed weight, and 0.22 for lodging when using inter-population predictions. This highlights potential challenges of using prediction for complex quantitative traits in populations and environments different from those used for training. All testing populations should also be represented in the training population to enhance the prediction accuracy of GS. Moreover, GS accuracy of winter injury in wheat in five locations ranged from 0.26 to 0.78 in a study by Beil et al. (2019). The study found that incorporating both major and minor genetic factors of cold tolerance into GS models improved accuracy for identifying genotypes adapted to the northern region. Modeling for population heterogeneity also improved prediction accuracy for winter survival in a previous study (Poudel et al., 2019). While GS can serve as a valuable selection method, efforts on increasing prediction accuracy should also be made.

5.4 | Assessing damage: Visual assessment

Visual assessment is a straightforward and widely used method of assessing cold damage and cold tolerance in field and controlled environment studies (Dumont et al., 2009; Wiering et al., 2018). Visual symptoms are correlated with physiological and biochemical changes as a result of low-temperature damage (Theocharis et al., 2012). Common symptoms at vegetative stages in susceptible plants include poor germination and emergence, stunted seedlings, leaf chlorosis, wilting, tissue necrosis, and reduced root nodulation (Maqbool et al., 2010; Perkus, 2018; Shafiq, 2012). Symptoms in susceptible plants at the reproductive stage include delayed heading and flowering, sterile pollen, and reduced seed yield (Thakur et al., 2010). On the other hand, winter hardy plants exhibit prostrate growth, increased branching, reduced height, low leaf area before winter, high chlorophyll content after winter, delayed flowering, and more growth in the fall under cold conditions (Klein et al., 2014; Lejeune-Hénaut et al., 2008; Xiong et al., 2007). Visual assessment of winter hardiness is generally conducted by developing a scale for one or more visual signs. It is done in a similar way in all species, with modifications to fit the specific symptoms and severity of damage in each species and for each environmental context. For example, Annicchiarico and Iannucci (2007) scored low-temperature damage in peas from one to nine, with one as no damage and nine as more than 75% mortality. Wiering et al. (2018) scored winter survival of hairy vetch in a controlled environment study on an individual plant basis, with zero as dead and one as alive. Plants that regrew after the freezing test or had any visible green pigment within photosynthetic tissue were considered alive in their study. Vann et al. (2019) assessed cold tolerance in winter pea, crimson clover, and hairy vetch on a whole-plot basis on a 0–5 scale, with 0 corresponding to complete death and 5 corresponding to no visual low-temperature injury. Each of these assessments is based on different phenotyping methods and can lead to different conclusions, especially when survival and injury do not correlate strongly. Reliance on individual judgement and lack of standardization in the protocol leading to inconsistent or inaccurate evaluations are drawbacks of the visual assessment method.

5.5 | Assessing damage: LT50

LT50, which represents the lethal temperature at which 50% of plants are killed, has been widely used to assess cold tolerance (Meyer & Badaruddin, 2001; Skinner & Garland-Campbell, 2008). It is primarily used in controlled environment studies (Michel et al., 2019; Sutka, 1981) and has been a valuable tool for identifying and comparing low-temperature tolerant accessions. Wiering et al. (2018) reported that the LT50 values established from freezing tolerance evaluation were highly correlated to their field winter survival data. LT50 values ranged from −4.5 to −8.5°C for peas (Meyer & Badaruddin, 2001; Murray et al., 1988), −6.3 to −7.4°C for forage legumes (Meyer & Badaruddin, 2001), −8.4 to −16.0°C for hairy vetch (Wiering et al., 2018), and −16 to −18°C for crimson clover (Hazrati et al., 2019) in previous studies. LT50 can vary by growth stage of the plant and by cultivar within the species (Wiering et al., 2018). It can be effectively used in both field and controlled environment studies.

5.6 | Assessing damage: Chlorophyll fluorescence assay and imaging

Cold stress can have both direct and indirect effects on photosynthesis (Rapacz & Woźniczka, 2009). Chlorophyll fluorescence assays (using a fluorimeter) and chlorophyll fluorescence imaging (using a camera) are widely used to assess photosynthesis efficiency under cold in a rapid and reproducible manner (Fracheboud et al., 1999; Legendre et al., 2021). Both methods calculate chlorophyll fluorescence parameters indicative of photosynthesis efficiency, such as the maximum quantum yield of photosystem II, ratio of variable fluorescence (Fv) to maximum fluorescence (Fm), and steady-state fluorescence normalized to minimal fluorescence (Legendre et al., 2021; Mishra et al., 2011). For both methods, leaves are adapted to the dark for around 20 minutes before chlorophyll fluorescence is measured. Detailed methods are described by Moustaka and Moustakas (2023), Ehlert and Hincha (2008), and Rizza et al. (2001). Chlorophyll fluorescence assays are performed using a small leaf area. It is unclear whether freezing damage develops homogeneously over the whole leaf area, making chlorophyll fluorescence assay measurements potentially prone to large errors (Ehlert & Hincha, 2008). Chlorophyll fluorescence imaging can be done at the cellular, leaf, and whole plant scale, providing more holistic stress detection (Pérez-Bueno et al., 2019). Chlorophyll fluorescence imaging (Dong et al., 2019) and fluorescence assays (Hakam et al., 2000; Su et al., 2015; Walker et al., 2019) have been applied to detect cold stress in many species. Georgieva and Lichtenthaler (1999) found 4˚C as the temperature limit for cold acclimation in winter pea through chlorophyll fluorescence assay. These parameters can detect cold effects and differentiate tolerant versus sensitive plants (Ehlert & Hincha, 2008). They have been employed to detect plant stresses before any visible signs of damage become apparent and thus could be used in winter annual cover crops for early detection of winter damage and cold stress. These methods could also be more useful when there are no visual differences between the plants. They are quick, inexpensive, and suitable for early stress detection method in large-scale experiments (Ehlert & Hincha, 2008).

5.7 | Assessing damage: Electrolyte leakage assay

The phenomenon of increased electrical conductivity in injured plant tissues is well-known and has been widely used to evaluate plant tolerance to abiotic stresses including cold tolerance (Dexter et al., 1932; Hannah et al., 2006; Sulc, Albrecht, & Duke, 1991; Sulc, Albrecht, Palta, et al., 1991). Electrolytes are contained within the plant cell membranes, which are susceptible to environmental stresses. Low temperatures induce changes in plant metabolism and physiology, such as reduced enzymatic activity and decreased photosynthetic capacity, which are often associated with an increase in membrane permeability and loss of membrane integrity (Campos et al., 2003; Dubey, 1997). Unstressed plant cells maintain electrolytes within the membrane, while stressed cells with damaged membranes leak electrolytes, such as K^+ ions, into apoplast. The amount of leaked ions from tissues is measured as the conductivity of tissue-imbibed water, and a higher conductivity indicates a more severe damage to membranes (Mattsson, 1996; McNabb & Takahashi, 2000). Electrolyte leakage is calculated as the percentage of conductivity of tissue imbibed water to the total conductivity from tissue after boiling (Hatsugai & Katagiri, 2018). Conductivity from a leaf sample can be measured with a conductivity meter, which can be portable. This is potentially a good method of assessing cold tolerance in winter annual legume cover crops. It can also serve as an early detection tool for stress, similar to chlorophyll fluorescence methods. However, this method is more laborious compared to chlorophyll fluorescence imaging and is hard to apply at a large scale (Ehlert & Hincha, 2008). Moreover, secondary damage to the plant tissue as a result of incubation in distilled water can result in less accurate measurement compared to chlorophyll fluorescence imaging (Ehlert & Hincha, 2008). Overall, electrolyte leakage assay is a simple, cost-effective, and widely used method for early detection of cold stress in winter annual legumes.

6 | APPROACHES TO CANDIDATE GENE IDENTIFICATION AND BREEDING FOR COLD TOLERANCE IN LEGUME COVER CROPS

CCB is a relatively new endeavor, with limited development of regionally adopted cover crop cultivars and widespread use of VNS seed. Many species used as cover crops are also used as forage or grain crops and have received varying degrees of breeding investment, although some trait targets are different for cover versus cash crop uses. Several efforts have been made in breeding and developing winter annual cover crop cultivars. For instance, the USDA NRCS (1981) identified the cereal rye cultivar Aroostook as a good cultivar for cover crop use. Another cereal rye cultivar, ND Gardner, was released in 2019 as a cover crop cultivar with excellent winter hardiness and high early season vigor and growth (Zwinger, 2019). Various winter annual legume cultivars have been introduced for multiple purposes, including feed, forage, and cover crops. Notable releases include hairy vetch cultivars, such as AU Early Cover, Americus, AU Merit, Purple Bounty, and Purple Prosperity; crimson clover cultivars, such as AU Robin, AU Sunrise, and Kentucky Pride; winter pea cultivars like Lynx, Wyowinter, Survivor, and Windham; and balansa

clover cultivars, such as Fixation and Frontier (Moore et al., 2020a; McPhee et al., 2007; Poffenbarger et al., 2023). With increased adoption of cover crops in recent decades, there has been increasing interest and investment in developing cover crops in both the public and private sectors. The CCB network initiated breeding of several winter annual legume cover crops in 2015, representing the largest public effort focused on CCB to date. Some private seed companies, such as Green Cover and GO Seed, along with several farmer breeders and universities, have released winter annual legume cultivars that are now widely used for cover crop purposes. For instance, the University of Wyoming released the winter pea cultivar Wyowinter (Homer et al., 2019), and GO Seed released the crimson clover cultivar Kentucky Pride (GO Seeds, 2021).

Compared to crops with longer histories of breeding investment, there are numerous areas in need of improvement, but developing winter hardy annual legume cover crops is a high priority. Approaches to identifying candidate genes and breeding winter hardy annual legume cover crops are described below.

6.1 | Hairy vetch

Breeding work has been initiated in hairy vetch including a focus on winter hardiness by the CCB network, but as of now, quantitative trait locus (QTL) mapping for cold tolerance has not been conducted in hairy vetch. As with many cover crops and other minor crop species, genetic resources for hairy vetch have historically been limited. However, a reference assembly for hairy vetch has been prepared (Fuller et al., 2023), and some molecular work has been conducted in this species, such as pooled DNA sequencing to identify QTLs for pod dehiscence and seed dormancy (Tilhou et al., 2024) and transcript profiling to identify genes for seed dormancy (Ali et al., 2023). Hairy vetch cultivar Purple Bounty was selected for winter survival and vigorous growth at Beltsville and Keedysville, MD (Moore et al., 2020a). This is currently the only cultivar development for winter survival reported in hairy vetch. Given the newly available genomic resources for hairy vetch, many opportunities exist to improve winter hardiness in this crop by using molecular tools and designing breeding pipelines to leverage selection for cold tolerance in breeding, trialing, and seed production environments.

6.2 | Winter pea

Efforts have been made to map QTLs and identify genomic regions associated with cold tolerance in winter pea. Dumont et al. (2009) identified six QTLs for cold tolerance in peas. The QTLs located in linkage groups V and VI were consistently identified in both field and controlled environment experiments, explaining 6.5%–46.5% of the phenotypic variance in their study. Klein et al. (2014) conducted a similar study and discovered three QTLs for cold tolerance primarily clustered in linkage groups III and VI. Linkage group III highlighted major developmental genic loci *Hr* and *Le*, and linkage group VI explained up to 71% of the variation in low temperature damage, suggesting that specific architecture and flowering ideotype define cold tolerance in winter pea (Klein et al., 2014). Lejeune-Hénaut et al. (2008) also reported the location of major QTL in the vicinity of the *Hr* locus, which was verified by findings of Klein et al. (2014). The *Hr* gene controls photoperiod response (Weller et al., 2012), providing a cold avoidance by delaying the reproductive growth until longer days when low temperature risk is lower. QTLs for raffinose metabolism and RuBisCO activity were colocalized with QTLs for low temperature damage, suggesting that their effect on photosynthesis might play a significant role in cold acclimation of peas (Dumont et al., 2009). The QTLs identified so far from different studies can serve as targets for marker-assisted selection in winter pea for cold tolerance (Lejeune-Hénaut et al., 2008). QTL identified from other molecular studies could also be used for molecular breeding in peas. A pea reference genome has been assembled (Kreplak et al., 2019; Yang et al., 2022), and a linkage map for peas has been constructed (Ellis et al., 1992; Sun et al., 2014). Klein et al. (2014) also developed a genetic map of peas, comprising 679 markers distributed across seven linkage groups, covering 947.1 cM. Nuclear genome size has been estimated for several pea accessions using various methods, and a size of 9.09 pg DNA/2C corresponding to the haploid genome size (1C) of 4.45 Gbp was estimated (Doležel & Greilhuber, 2010).

Despite progress in mapping genes and QTLs governing cold stress, breeding of northern region-adapted peas has been limited. Some selection efforts have been made, and winter hardy pea cultivars developed for forage or grain use have been released, such as Windham (McPhee et al., 2007), Lynx (McGee et al., 2013), and Wyowinter (Homer et al., 2019). Inconsistent winter survival of current cultivars in the northern United States suggests that more efforts are needed for developing winter hardy pea cultivars.

6.3 | Crimson clover

To date, QTL mapping for cold tolerance has not been performed in crimson clover. Some cultivars of crimson clover, such as Lana (Hollowell, 1960), Frontier (Knight, 1963), KY C-l crimson clover germplasm (Taylor, 1986), and Kentucky Pride (GO Seeds, 2021), have been selected for improved winter growth and winter hardiness. Other cultivars such as Dixie, AU Robin, AU Sunrise, AU Sunup, and Contea were selected and released for superior agronomic characteristics, earlier maturity, and high forage yield (Hollowell, 1953;

Poffenbarger et al., 2023; Smith, 2010; Van Santen et al., 1992). However, the current cultivars have inconsistent winter survival in the northern regions. Breeding efforts have been initiated by the CCB network, the Auburn University, and the University of Florida to improve winter survival and other traits in crimson clover for forage and cover crop use. More efforts to screen accessions, construct linkage maps, map QTL, and breed winter hardy cultivars are needed in crimson clover to improve winter hardiness. Moreover, current North American and European crimson clover cultivars were developed from a limited genetic base (Steiner et al., 1998). Exploring genetic diversity could provide a valuable source of cold tolerance and other important traits.

So far, studies on mechanisms of cold tolerance, selection, breeding, and QTL mapping have been limited in most winter annual cover crops including hairy vetch and crimson clover. While similar mechanisms of winter hardiness and cold acclimation exist among winter annual cover crops, investigating individual species is still needed to reveal potential species-specific features and to accelerate the improvement of winter hardiness.

7 | ENVIRONMENTAL AND MANAGEMENT FACTORS AFFECTING COLD TOLERANCE

The winter hardiness of winter annual legume cover crops is influenced by the growing location, with differing temperature fluctuation, soil type, and snow accumulation (Gatiboni & Reberg-Horton, 2021; Shafiq, 2012).

Management practices play an important role in maximizing soil coverage and increasing ecological benefits from these cover crops (Carmona et al., 2022). Appropriate management considerations may include planting date (Carmona et al., 2022) and cropping system (Jannink et al., 1997), while conducting multisite varietal trials can allow exploration of genotype × environment × management interaction for winter hardiness. Fall planting dates influence the developmental stage reached before the onset of winter conditions. Early planting of cover crops increases biomass cover, winter survival, and other ecosystem services obtained (Mirsky et al., 2009). While most winter annual cover crops perform better at earlier planting dates, some winter pea genotypes can transition from rosette to upright growth habit, leading to reduced winter survival, if planted too early. Winter pea lines can withstand winter conditions when they display rosette growth with small leaves and short internodes at the onset of winter and possess quantitative photoperiodic sensitivity (Urbatzka et al., 2012). Some guidelines on planting dates and management practices are available from the Northeast Cover Crop Council. However, the guidelines are limited and are region/species specific (Carmona et al., 2022), so identifying appropriate planting dates for these species can help to increase their winter survival and overall performance.

Intercropping with cereals has also been reported to improve the winter survival of legumes (Jannink et al., 1997; Vann et al., 2019). The presence of cereals offers several benefits, including improved weed suppression (Vann et al., 2019) and improvement in legume winter survival by reducing low-temperature heaving, buffering low-temperature extremes through greater snow cover retention, and reducing air movement at the soil surface (Jannink et al., 1997; Smith, 1975). However, the growth of cereals must be robust to protect legumes from cold damage in the cereal–legume mixture (Vann et al., 2019). When growing cover crops in the mixture, management practices need to be altered for the successful growth of each crop in the mixture. Hayden et al. (2015) planted a mixture of cereal rye and vetch at different planting dates and found that delaying rye seeding until vetch emergence increased vetch shoot biomass by 760–1060 kg ha^{-1} relative to co-seeding in earlier planting dates. However, in their study, later seeding of cereal rye did not benefit vetch biomass at later planting dates; instead, it led to a reduction in vetch winter survival by 12%–42% when compared with co-seeding. This underscores the importance of considering the impact of management practices on the winter survival and performance of mixtures, especially in the case of legumes in a legume–cereal mixture.

8 | FUTURE PROSPECTS

There are many opportunities and challenges in developing winter hardy annual legume cover crops. Studies of cold tolerance mechanisms have been limited, especially in crimson clover and hairy vetch. Plant species may differ in their capacities for cold tolerance, cold acclimation, deacclimation, and reacclimation treatments. Determining the lethal cold temperature, recognizing signs of various types of damage, and identifying tolerance mechanisms in these species are crucial for developing cold tolerant winter cover crops.

In addition, identifying genetic diversity, QTL mapping, and gene identification for cold tolerance can enable marker-assisted breeding and genomic prediction for hairy vetch and crimson clover, which have had little study so far, as well as for winter pea, where some efforts have been made.

Climate change makes it more challenging to develop winter hardy legume cover crops. Accessions and cultivars that are currently winter hardy may not be so in the future as the more frequent freeze/thaw cycles would reduce their cold tolerance, deacclimation resistance, and reacclimation capacity. Therefore, it is crucial to identify cultivars with high deacclimation resistance and reacclimation capacity for all winter annual legumes. With a diversity of species as potential cover crops, winter hardiness could also be explored in

other legumes, such as balansa clover and subterranean clover (*Trifolium subterraneum*), which may have hardier accessions compared to the legumes discussed above.

One of the main challenges in breeding cold tolerant cover crops lies in the lack of knowledge and limited resources for these crops compared to many cash crops. The development of cold hardy cultivars is needed for many, and it requires significant time and effort, especially for hairy vetch and crimson clover. The CCB network has made significant progress in selecting winter annual legume cultivars with superior winter hardiness compared to existing cultivars. Continued public and private breeding efforts, along with cover crop management research and incentives for cover crop use, are necessary for the development and use of winter hardy legume cover crops in the northern United States.

AUTHOR CONTRIBUTIONS

Raksha Thapa: Conceptualization; writing—original draft; writing—review and editing. **Solveig Hanson**: Writing—review and editing. **Jian Hua**: Writing—review and editing. **Virginia M. Moore**: Conceptualization; funding acquisition; supervision; writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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