The wheat Sr22, Sr33, Sr35 and Sr45 genes confer resistance against stem rust in barley

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Abstract

In the last 20 years, stem rust caused by the fungus Puccinia graminis f. sp. tritici (Pgt), has re-emerged as a major threat to wheat and barley production in Africa and Europe. In contrast to wheat with 60 designated stem rust (Sr) resistance genes, barley’s genetic variation for stem rust resistance is very narrow with only ten resistance genes genetically identified. Of these, only one complex locus consisting of three genes is effective against TTKSK, a widely virulent Pgt race of the Ug99 tribe which emerged in Uganda in 1999 and has since spread to much of East Africa and parts of the Middle East. The objective of this study was to assess the functionality, in barley, of cloned wheat Sr genes effective against race TTKSK. Sr22, Sr33, Sr35 and Sr45 were transformed into barley cv. Golden Promise using Agrobacterium-mediated transformation. All four genes were found to confer effective stem rust resistance. The barley transgenics remained susceptible to the barley leaf rust pathogen Puccinia hordei, indicating that the resistance conferred by these wheat Sr genes was specific for Pgt. Furthermore, these transgenic plants did not display significant adverse agronomic effects in the absence of disease. Cloned Sr genes from wheat are therefore a potential source of resistance against wheat stem rust in barley.

Introduction

Wheat stem rust, caused by the fungus P. graminis f. sp. tritici (Pgt), is one of the main threats to barley (Hordeum vulgare) production in North America (Steffenson, 1992) and Australia (Dill-Macky et al., 1991). This destructive fungal disease can cause a significant reduction in plant growth and yield of both barley and wheat (De Wolf et al., 2011). In 1999, a new virulent isolate of Pgt called Ug99 (typed as race TTKSK according to Jin et al., 2008) was detected in Uganda which had overcome Sr31, a widely deployed stem rust resistance gene in bread wheat (Triticum aestivum; Pretorius et al., 2000). At that time Ug99 and its derivatives were virulent on more than 80% of the world’s wheat cultivars (Singh et al., 2008). In recent years new Pgt races, that are not members of the Ug99 race group, have caused disease outbreaks on wheat in Europe (including Germany; Olivera Firpo et al., 2017, and Italy; Bhattacharya, 2017), Asia (Russia; Shamanin et al., 2016) and Africa (Ethiopia; Olivera et al., 2015).

Effective ways of controlling this disease include fungicide application and breeding for resistant cultivars (McIntosh et al., 1995), with this latter strategy being the most cost-effective and environmentally acceptable. However, when lines carrying a single resistance (R) gene effective against a specific disease are deployed, strong selection pressure is imposed on the pathogen population often leading to the development of mutants capable of overcoming the resistance and the outbreak of an epidemic (Stakman, 1957). Notwithstanding, there are a few cases where R genes effective against Pgt have shown remarkable durability despite being deployed as a single gene for many years over a wide area where the pathogen is prevalent. Examples of such durability include Sr31 which protected wheat from major losses for over 30 years until the Ug99 outbreak in 1999 (Ayliffe et al., 2008; Pretorius et al., 2000; Singh et al., 2006) and barley Rpg1, which has been widely deployed since the 1940s (Brueggeman et al., 2002). An alternative strategy is the simultaneous deployment of several R genes within a cultivar to prolong R gene efficacy in the field. There is no selective advantage for pathogen strains that have mutated to overcome a single R gene in such cultivars, thus imposing a barrier to the stepwise evolution of virulence (Dangl et al., 2013; Ellis et al., 2014; McDonald and Linde, 2002). However, it is difficult to ensure that multiple R
genes, which may be scattered throughout the genome, remain together in a breeding programme. None-the-less, genetic resistance to cereal rust diseases has been fundamental for crop protection. For more than 100 years, breeders have introgressed resistance into wheat by undertaking wide crosses between wheat and its wild or domesticated relatives. Notable examples include the transfer of the stem rust resistance genes Sr2 from emmer wheat (Triticum turgidum subsp. dicoccum; McFadden, 1930), Sr31, Sr50 and Sr15-Ampgo from rye (Secale cereale; Mago et al., 2005b), Sr24 and Sr26 from Thrinopyrum ponticum (Mago et al., 2005a), and Sr26 from T. tiggeheuer (McIntosh and Gyarfas, 1971). However, sexual incompatibility and long generation times can impose significant barriers to successful gene introgression (Erickson, 1945). Also, linkage drag of deleterious alleles has hindered the deployment of many Sr genes in wheat, for example, Sr22 and Sr42 due to yellow flour pigmentation and/or reduced yield and delayed heading date (Knott, 1984; Marais, 1992; Niu et al., 2014).

In contrast to wheat, where 60 stem rust resistance genes have been described (McIntosh et al., 2017; Chen, Guo et al., 2018), only ten stem rust resistance genes have been reported in barley; these being Rpg1 (Brueggeman et al., 2002; Powers and Hines, 1933; Steffenson, 1992), Rpg2 (Case et al., 2018; Patterson et al., 1957), Rpg3 (Case et al., 2018; Jedel, 1990; Jedel et al., 1989), Rpg4 (Jin et al., 1994), Rpg5 (Brueggeman et al., 2008; Sun and Steffenson, 2005; Sun et al., 1996), Rpg6 (Fetch et al., 2009), Rpg7 (Henningsen and Steffenson, 2018), Rpg8H (Steffenson et al., 1984; Sun and Steffenson, 2005), RpgU (Fox and Harder, 1995), and an undesignated one from accession Skinless (Luig, 1957). Rpg1 is the most widely deployed amongst these genes due to its broad-spectrum resistance which has remained effective for over 70 years (Brueggeman et al., 2002; Steffenson, 1992). However, a recent study showed that this gene is not effective against the Ug99 lineage race TTKSK (Steffenson et al., 2017). The rpg4-DMediated Resistance Locus (RMR3) is the most effective gene complex identified in barley against race TTKSK. It consists of two tightly linked loci: RMR1-1, containing the three genes of HvRga1, Rpg5, and HvAdf3, and RMR2 (Wang et al., 2013). In addition, seedling assays undertaken on two panels of 1,924 and 934 genetically diverse barley cultivars and wild barley accessions (H. vulgare subsp. spontaneum), showed that more than 95% and 97% of accessions respectively, were susceptible to race TTKSK. Hence, it is important to identify novel sources of resistance to safeguard barley from stem rust (Steffenson et al., 2017). Given the limited number of R genes available for Pgt protection in barley, interspecies R gene transfer is a potentially valuable alternative (Wulff and Moscou, 2014).

The majority of R genes cloned encode proteins containing nucleotide-binding and leucine-rich repeat domains (NLR proteins; Kourelis and van der Hoorn, 2018). Plant genomes typically contain several hundred NLR genes (Baggs et al., 2017). NLRs detect the presence of a pathogen by recognising the presence of pathogen effector molecules. This recognition can be direct, although more often it is indirect whereby the NLR (also known as the ‘guard’) recognises the effector-mediated modification of a host pathogenicity target or a ‘decoy’ of this target, (also known as the ‘guardee’) (van der Hoorn and Kamoun, 2008; Dodds and Rathjen, 2010; Kourelis and van der Hoorn, 2018). In some cases, the decoy pathogenicity target is integrated into the NLR itself—such NLRs (dubbed ‘sensor’ NLRs) often work in concert with a second ‘helper’ NLR which initiates downstream signalling upon activation of the sensor NLR (Cesari et al., 2014). NLR proteins

that function by either mechanism have been successfully transferred by transgenesis to distantly related, nonsexually compatible species and shown to function in some instances. For example, the L6 protein of flax (Linum usitatissimum, a member of the Linaceae) directly binds a corresponding AvrL567 effector protein of the flax rust pathogen Melampsora lini. When the L6 gene is co-expressed with AvrL567 in Nicotiana benthamiana (a member of the Solanaceae) a hypersensitive resistance response is activated (Dodds et al., 2004). Similarly, a number of R genes that function by guardee recognition have been shown to function upon interspecies transfer, exemplified by the transfer of the Arabidopsis thaliana (a Brassicaceae) guard and guardee gene pairs RPS2 or RPM1 with RNA4 (Chung et al., 2011; Day et al., 2005) and RPS5 with PBS1 (Ade et al., 2007) into N. benthamiana. Finally, the Arabidopsis paired Rns1/Rps4 sensor/helper NLRs support functional intra-family transfer within the Brassicaceae as well as inter-family transfer to the Solanaceae (N. benthamiana and Solanum lycopersicum) and Cucurbitaceae (Cucumis sativus) (Narasuk et al., 2013).

Transferring R genes between species by conventional crossing can be a tedious task due to the extensive backcrossing usually required. However, it is now relatively straightforward to introduce these R genes as transgenes by transformation thereby avoiding this breeding requirement. Further advantages of transgenesis include that transfer is not limited to sexually compatible species, there is no linkage drag, and it becomes possible to stack multiple R genes at the same locus to ensure co-inheritance. When transferred between different species and families, these R genes can function normally (reviewed in Wulff et al., 2011) and agronomically important examples include the B52 gene from pepper (Capsicum annuum), which was successfully transferred to tomato (S. lycopersicum), another Solanaceous species, where it confers resistance to bacterial leaf spot (Tai et al., 1999) and CcRps1 from pigeonpea (Cajanus cajan), which confers resistance to Asian soybean rust when introduced into soybean (Glycine max; Kawashima et al., 2016).

Barley (H. vulgare) and wheat (T. aestivum) diverged from a common Triticeae ancestor approximately 10 to 14 million years ago (Schlegel, 2013; Figure 1a). It is therefore likely that wheat NLR genes will function in barley, and that wheat Sr genes could be used to improve the resistance of barley to Pgt. Nine NLR-encoding Sr genes have been cloned so far from wheat or its wild progenitors: Sr13 from durum wheat (T. turgidum ssp. durum; Zhang et al., 2017), Sr21, Sr22 and Sr35 from T. boeoticum and T. monococcum (Chen et al., 2018; Saini et al., 2013; Steurnergel et al., 2016), Sr33, Sr45, Sr46 and SrTA1662 from Aegeilops tauschii (Arora et al., 2019; Periyannan et al., 2013; Steurnergel et al., 2016), and Sr50 from rye (Secale cereale; Mago et al., 2015; Figure 1b). All these genes confer resistance to the Ug99 race group.

In the coming years, it is anticipated that there will be a large increase in the number of cloned Sr genes due to the development of rapid R gene isolation methods such as TACCA (Third et al., 2017), MutRenSeq (Steuernagel et al., 2016), MutChromSeq (Sanchez-Martín et al., 2016) and AgRenSeq (Arora et al., 2019). Often the functional testing of R gene candidates is delayed by the need to isolate native regulatory sequences and to assemble large binary constructs encoding the R gene. This process can be accelerated by substituting regulatory elements from the previously cloned R genes, and generating R gene constructs using the type IIS restriction endonuclease-based Golden Gate cloning technique (Engler et al., 2008). Further,
the incorporation of type IIS restriction sites allows the generation of user-defined overhangs thereby enabling simultaneous cloning of multiple fragments. This assembly method has dramatically decreased the amount of time required to design and develop gene constructs. However, one major requirement is that the fragments to be assembled must be free from recognition sites of the selected type IIS restriction endonuclease. This requires ‘sequence domestication’ (removal of internal type IIS sites). While the open reading frame can be maintained due to the redundancy in the genetic code, the removal of sites from the regulatory sequences (introns, promoter and terminator) may affect gene expression and function.

In this study, we generated constructs encoding the wheat Sr22, Sr33, Sr35 and Sr45 genes using Golden Gate cloning and transformed these into barley (Figure 1b). The resultant transgenic barley lines showed high levels of resistance to Pgt race MCCFC. The homozygous lines showed near immunity to this Pgt isolate (Figure 3a and Table S2) whereas null lines and Golden Promise control seedlings all showed extensive Pgt growth.

Four T2 lines, each derived from an independent transgenic event, were selected that were homozygous for the Sr33 transgene and T2 families (11-12 seedlings) from each line inoculated with Pgt race MCCFC and TKTTF. All seedlings tested showed resistance to both races of Pgt (Figure 3b and c; Table S3). In addition to susceptible Golden Promise control seedlings, additional wheat control lines were included. The Sr33-containing cv. Chinese Spring and an EMS-derived mutant carrying a non-functional allele of Sr33 (Periyannan et al., 2013) were used as resistant and susceptible controls, respectively, to demonstrate the avirulence of Pgt race MCCFC to Sr33 (Table S3).

The wheat Sr22, Sr35 and Sr45 genes can be sequence-modified but retain gene function

A functional wheat NLR transgene is typically 10 kb in length and consists of 4 kb of 5’ and 3’ regulatory elements, 3 kb of exons and 3 kb of introns. These long, contiguous sequences can be difficult to isolate and verify from a non-reference hexaploid wheat genome, and their synthesis is expensive. Multi-segment Golden Gate assembly (Weber et al., 2011) was therefore tested as an alternative for rapid and cost-effective generation of full-length Sr gene constructs using either native or non-native regulatory sequences. Firstly, the effect of sequence

Figure 1 Strategy for improving barley resistance to stem rust with cloned wheat Sr genes. (a) Wheat and barley diverged from a common Triticeae ancestor 10-14 million years ago. (b) Cloned Sr genes from wheat, rye and the domesticated and wild relatives of wheat (coloured circles), function when transformed into barley (black outline, this study). (c) The future stacking of multiple cloned Sr genes in barley may provide durable resistance to wheat stem rust. At, Aegilops tauschi; Td, Triticum turgidum ssp. durum; Tb, Triticum boeoticum; Tm, Triticum monococcum; Sc, Secale cereale. [Colour figure can be viewed at wileyonlinelibrary.com]
domestication (i.e. the removal of all Type IIIS Bsal and Bpi restriction enzyme sites) was examined on Sr33 function. Four Bpi sites were removed from the Sr33 promoter while three Bpi and two Bsal sites were removed from the Sr33 open reading frame (Figure 2c). Although the Sr33 open reading frame was faithfully maintained through domestication, there was a risk that the removal of the four Bpi sites in the 5' regulatory sequence would disrupt gene function. The domesticated full-length Sr33 gene (Sr33d) was transformed into cv. Golden Promise. In total, five homozygous and five null lines were selected from two segregating T1 families and infected with Pgt race MCCFC. All five homozygous lines conferred resistance while the five null lines showed susceptibility (Figure 3d and Table S4). These data confirm that Sr33d encodes functional Sr33 resistance in spite of the sequence domestication process.

Having demonstrated that the endogenous wheat genes Sr22 and Sr33 can provide Pgt resistance in barley and that a sequence-modified version of Sr33 (Sr33d) maintains function, two additional domesticated wheat Sr genes were developed. A domesticated Sr35 construct (Sr35d) was generated by multi-segment Golden Gate assembly which removed seven Bpi sites from the gene (Figure 2d). In total, eight homozygous and nine null lines were selected from three Sr35d segregating T1 families and tested with Pgt race TKTTF. All eight homozygous lines showed a high level of resistance while the nine null lines were all susceptible (Figure 3e and Table S5). A chimeric Sr45 gene construct (Sr45d) was also assembled by Golden Gate and consisted of a Bsal domesticated Sr45 open reading frame flanked by Sr33 5' and 3' regulatory sequences (Figure 2e). This construct did not require the removal of Bpi sites from Sr33 regulatory sequences; however, the assembly resulted in the introduction of four additional nucleotides at both the junction between the Sr33 promoter and start codon of Sr45 and the termination codon of the Sr45 ORF and the Sr33 3' regulatory sequence (Figure 2e). In spite of these modifications all seven homozygous lines selected from three Sr45d segregating T1 families showed a very high level of resistance to Pgt race MCCFC (Figure 3f and Table S6). These data demonstrate that these wheat NLR genes can be sequence-modified to facilitate further molecular biological manipulation and that regulatory sequences can be functionally exchanged between NLR genes in some instances.

Pathogen and race-specific resistance is maintained by wheat Sr genes in barley

To rule out the possibility that these resistant transgenic barley lines are a consequence of an ectopic non-specific defence reaction, we tested these transgenic plants with the barley leaf rust pathogen Puccinia hordei. All Sr22, Sr33, Sr33d, Sr35d and Sr45d transgenic barley lines, as well as Golden Promise was susceptible or moderately susceptible to P. hordei. In contrast, a barley control, accession PI584760 carrying Rph14, and the wheat Sr33 line, which is a non-host of P. hordei, were both resistant indicating that the barley resistance observed in stem rust infection assays was specific to Pgt (Figure S1; Table S7-S11). Ten Sr33d transgenic T1 families were also tested with Pgt race MCCFC (virulent to Sr33) including the three T2 lines described above that are resistant to Pgt race TKTTF. All ten Sr33d T1 families were intermediate to susceptible to Pgt race MCCFC (Table S12) indicating that race specificity of this wheat gene is maintained in transgenic barley.

Expression of wheat Sr genes does not affect agronomic traits in barley

To determine whether expression of wheat Sr genes in transgenic barley affects agronomic traits, we selected homozygous lines of Sr22, Sr33d, Sr35d and Sr45d and compared plant growth and seed characteristics to non-transgenic Golden Promise in the absence of disease (Figures 4 and 5). Statistical analyses revealed that none of the homozygous Sr gene transgenic lines differed significantly from the null lines with regard to the agronomic traits measured including tiller number (Figure 4e and Table S13) thousand grain weight (Figure 5 and Table S14), and growth rate (Figure S2; Table S15-S18).

![Figure 2: Schematic overview (not shown to scale) of the constructs described in this study. Binary construct containing (a,b) full-length Sr22 and Sr33, respectively, driven by their 5' and 3' native regulatory elements, (c) Bsal and Bpi domesticated full-length Sr33d driven by its 5' and 3' native regulatory elements, (d) Bpi domesticated full-length Sr35d driven by its 5' and 3' native regulatory elements and (e) Bsal domesticated Sr45d driven by 5' and 3' Sr33 regulatory elements. The non-native four-nucleotide linker in pBW_0141 was introduced immediately before the start codon (underlined) and immediately after the stop codon (underlined). Grey arrows correspond to the removed Bpi sites and white arrows correspond to the removed Bsal sites. Sites removed in the coding regions were achieved by introducing synonymous mutations. Blue and green rectangles correspond respectively to hptII and nptII plant selectable marker genes. [Colour figure can be viewed at wileyonlinelibrary.com]]
Discussion

Barley is a major food staple in the mountainous areas of Central Asia, Southwest Asia and Northern Africa (Von Bothmer et al., 2003). The re-emergence of wheat stem rust as a major biotic constraint to wheat production also poses a threat to barley production. A recent study revealed very limited resistance to the Ug99 lineage race TTKSK in both cultivated barley and its immediate progenitor H. vulgare ssp. spontaneum (Steffenson et al., 2017). This Pgt isolate has caused major epidemics in East Africa since 1999. One avenue for improving resistance to stem rust in barley is to utilise diverse genetic resistance from outside the barley gene pool.

R genes typically function when transferred from one species to another within the same family (Wulff et al., 2011). In this study, the wheat Sr22, Sr33, Sr35 and Sr45 genes were shown to function when transferred to barley and confer race-specific disease resistance to Pgt. Recently, the wheat Sr35 and Sr50 have been demonstrated to be functional when transiently expressed in barley (Bolus et al., 2020; Saur et al., 2019). Other examples of R gene transfer in monocots include the introduction of the maize NLR gene Rxo1 into rice where it confers resistance to bacterial streak disease (Zhao et al., 2005) and single-cell transient expression assays of the barley Mla6 gene in wheat where it confers AvrMla6-dependent resistance to Blumeria graminis f. sp. hordei (Bgh) (Halterman et al., 2001). In concordance, the wheat Sr22, Sr33, Sr35 and Sr45 genes also function in barley, suggesting that the downstream signalling pathway(s) of NLR proteins in wheat and barley has remained conserved since the divergence of these two species 10 to 14 million years ago (Schlegel, 2013).

The functional transfer of these wheat Sr genes into barley potentially provides additional sources of stem rust resistance in this recipient species. Interestingly, in most cases, these barley transformants displayed a highly resistant reaction that is stronger than that observed for the endogenous wheat genes. Similarly, when the barley Rpg1 gene was expressed as a transgene in barley, this also gave rise to a near-immune reaction (Horvath...
In contrast to these transgenic barley experiments, near-immune reactions were extremely rare when large scale screening of wild and cultivated barley lines was undertaken using different Pgt races (e.g. Steffenson et al., 2017). The increased resistance conferred by these transgenes may be a consequence of elevated expression arising from position effects or alternatively their interaction with a new genetic background in the case of interspecies transfer.

Importantly, we confirmed that race specificity of the Sr35 gene was maintained in barley. We assume this is also the case for Sr22, Sr33 and Sr45, but cannot confirm this due to an absence of Pgt races virulent on both Golden Promise and towards these genes. Unlike most barley cultivars, Golden Promise does show resistance to many Pgt races which makes Sr gene analysis difficult as it is one of the few transformable barley cultivars available. However, for these latter genes, we confirmed that resistance is not a consequence of a non-specific defence reaction caused by ectopic expression of these genes. All transgenic barley lines tested with P. hordei, the causal agent of barley leaf rust, were as susceptible as the Golden Promise control lines demonstrating species-specific resistance conferred by these Sr genes. The phylogenetic relatedness of these two fungal pathogen species argues against a generic defence response being activated. Interestingly these data also suggest that there is little conservation of effectors recognised by these wheat Sr genes in P. hordei.

Transgenic plants displaying a resistance phenotype derived from a transgene have been reported to induce ectopic expression of downstream defence genes in the absence of the pathogen (Mindrinos et al., 1994; Oldroyd and Staskawicz, 1998), which may affect plant growth and development. Such precocious triggering of defence responses could result from inappropriate interaction between NLR proteins and the pathogenicity targets they guard (Kruger et al., 2002). We showed that there was no significant difference between barley transgenic homozygous and null lines of the four Sr genes with respect to agronomic traits such as tiller number, growth progression, or thousand grain weight (TGW) (Figures 4 and 5; Figure S2; Table S13-S18), indicating that ectopic defence activation by the wheat Sr genes in barley was absent or minimal. In contrast, expression in barley of the wheat leaf rust resistance gene Lr34, which encodes an abscisic acid transporter (Krattinger et al., 2019), provided resistance to multiple pathogens (Risk et al., 2013), concomitant with severe necrosis and stunted growth (Chauhan et al., 2015). In this case, pathogen resistance and necrosis could be uncoupled by co-expression of a susceptible allele of Lr34 (Chauhan et al., 2015).
In the last few years, many significant improvements have been made in the field of R gene cloning. For example, sequence comparison of multiple independently-derived mutants, facilitated by various genome complexity reduction technologies, for example, NLR exome capture (Steunagem et al., 2016) or chromosome flow sorting (Sánchez-Martín et al., 2016; Third et al., 2017) was used to rapidly clone Sr22, Sr45, Pm2 and Lr22a from hexaploid wheat. Recently, the requirement of mutagenesis was overcome by combining association genetics with NLR exome capture on a diversity panel of Ae. tauschii (Arora et al., 2019). The resulting application, AgRenSeq, allowed the rapid cloning of Sr46 and identification of a high confidence candidate for SrTA1662 (Arora et al., 2019). These advances coupled with the recent availability of a wheat reference genome will greatly accelerate R gene discovery and cloning (Md. Hatta et al., 2019).

As more wheat R genes are cloned, they can be tested in barley using the strategy demonstrated in this paper and, in the case of stem rust potentially provide greater control of the disease in this species. The ability to modify these R gene sequences by multi-segment Golden Gate assembly (Weber et al., 2011), even including regulatory elements, and yet maintain gene function will greatly facilitate the manipulation and validation of these genes. Unlike hexaploid wheat, the diploid nature of barley will help contribute to our understanding of the fundamental aspects of wheat stem rust resistance. Its greater amenability to mutagenesis will enable the identification of additional genes required for rust R gene function, as well as potential host susceptibility genes. Interestingly a higher proportion of rust R genes in barley are recessive (26.3%) compared to wheat (6.7%) (Uauy et al., 2010). The cloning of such recessive resistance genes may provide novel fundamental insight into plant pathogen interactions.

NLR genes are not the sole means of generating disease resistant plants. Another approach to improve wheat stem rust resistance in barley is to combine multiple, additive minor effect quantitative trait loci (QTLs). Bi-parental and genome-wide association studies (GWAS) have identified QTLs associated with stem rust resistance in barley (Case et al., 2018; Mamo, 2013; Sallam et al., 2017; Turuspekov et al., 2016; Zhou et al., 2014). More recently, GWAS on adult plants identified seven novel QTLs associated with adult plant resistance to Pgt race QCCJB and a composite of races TTKSK, TTKST, TTKTK and TTKTT, which are all members of the Ug99 lineage (Case et al., 2017). The presence of adult plant resistance (APR) genes or minor effect QTLs has been shown to enhance the strength of race-specific R genes (Hiebert et al., 2016) and promote their longevity (Brun et al., 2010).

Interestingly an APR gene has also been transferred between monocot species by transformation and shown to function. The wheat Lr34 APR gene has been shown to provide resistance against multiple, diverse rust, mildew and blast fungal pathogens in barley, rice, durum wheat, maize and sorghum (Krattinger et al., 2016; Rinaldo et al., 2017; Risk et al., 2013; Schnippekoetter et al., 2017; Sucher et al., 2017), although the mechanism of this resistance is as yet unknown. Wide interspecies transfer of functional disease resistance is therefore not limited to NLR genes.

Given that wheat R genes function in barley, it is reasonable to expect that barley R genes will also function in wheat. Therefore, barley R genes conferring resistance to wheat stripe rust (P. striiformis f. sp. triticum) (Dawson et al., 2016) might be deployed in wheat to control this disease. However, caution should be taken so that R genes transferred from one crop to another are not easily overcome which would potentially facilitate a host jump and create a new disease problem. Ideally, R genes with different specificities should be combined as a multi R gene stack (Figure 1c), preferably with the inclusion of APR genes. This is likely to confer more durable resistance by delaying the emergence of resistance-breaking strains of the pathogen (Dangl et al., 2013; McDonald and Linde, 2002).

In summary, functional transfer of the Sr22, Sr33, Sr35 and Sr45 genes into barley has created a new source of resistance to stem rust in barley. As more novel rust R genes are cloned and shown to function in barley, these could subsequently be deployed in a stack to provide broad-spectrum resistance and reduce the risk of resistance breakdown. Future field experiments with transgenic barley plants expressing single or multiple Sr transgenes will be useful to assess the agronomic value of wheat Sr genes in barley cultivation.

**Experimental procedures**

**Generation of binary constructs carrying Sr genes**

To assemble a plant transformation construct containing an Sr22 expression cassette, a 9855 bp fragment of DNA containing the Sr22 coding sequence, 2377 bp of 5’ regulatory sequence (i.e. 5’ of the predicted start codon) and 1560 bp of 3’ regulatory sequence (i.e. 3’ of the STOP codon) was synthesised by a commercial DNA synthesis provider (Life Technologies Ltd) with flanking NotI sites. The synthetic DNA was cloned into the NotI site of the pVec8 binary vector (Wang et al., 1998).

The Sr33 gene sequence from the binary vector pVecNeo + Sr33 (Periyannan et al., 2013) was introduced into the binary vector pWBvec8 (Steunagem et al., 2016) using PspOMI and NotI restriction enzymes. The Sr33 gene sequence in the later construct pWBvec8 + Sr33 was proof read using ATMSF1, ATMSR1, ATMSF2, ATMSR2, ATMSF3 and ATMSR3 primers (Periyannan et al., 2013).

To generate Sr33d, a 7854 bp fragment of Sr33, including 2381 bp of 5’ and 1405 bp of 3’ regulatory sequence and an 8255 bp fragment of Sr25d, including 2462 bp of 5’ and 2615 bp of 3’ regulatory sequence was synthesised flanked by a pair of divergent Bpi recognition sites. Prior to synthesis, any recognition sequences for the restriction endonucleases BsaI and BpiI were removed by introducing synonymous mutations in coding sequences and avoiding intron splice junctions. This fragment was simultaneously assembled using Golden Gate cloning (Weber et al., 2011) into a level two Golden Gate acceptor plasmid, pAGM4723 (Weber et al., 2011), with a hygromycin selectable marker cassette to confer resistance to hygromycin.

A transformation construct for barley containing an Sr45d expression cassette (with internal BsaI sites removed by introducing synonymous mutations) was constructed as described in Arora et al., (2019) and the assembled gene cassette was cloned into the NotI site of the pVec8 binary vector. All binary plasmids containing the desired insert were transformed into Agrobacterium tumefaciens (strain AGL1) for transformation of barley.

**Barley transformation**

Agrobacterium-mediated transformation of Sr gene constructs Sr22, Sr33d, Sr35d and Sr45d into barley cv. Golden Promise was performed as described in (Hinchliffe and Harwood, 2019). The number of transgene copies of Sr22, Sr33d, Sr35d and Sr45d in barley was determined by iDNA Genetics (Norwich, UK) using...
qPCR as described in Bartlett et al., (2008). In brief, a PCR ampiclon targeting the neomycin phosphotransferase II (nptII) or hygromycin phosphotransferase II (hptII) gene (with a 6-carboxyfluorescein; FAM reporter) and an ampiclon targeting a barley internal positive control (IPC, with a 2′-chloro-7′-phenyl-1,4-dichloro-6-carboxyfluorescein; VIC reporter) were amplified together in a multiplex reaction (15 min denaturation, 40 cycles of 95 °C for 15 s and 60 °C for 60 s) in an ABI7900 PCR machine. Fluorescence from the FAM and VIC fluorochromes was measured during each 60 °C step, and the cycle threshold (Ct) values obtained. The difference in Ct value between the nptII or hptII gene and the IPC was used to allocate the assayed samples into groups with the same gene copy number. Plants with a single copy and homozygous for the transgene were selected for phenotyping. The stabilised Sr22, Sr33d, Sr35d and Sr45d transgenic barley lines are available from the GRU SeedStor (https://www.seedstor.ac.uk/). Binary vector pWBvec8 + Sr33 was transformed into cv. Golden Promise using Agrobacterium-mediated transformation as described in Moore et al. (2015). Four advanced generation lines, SH1, SH2, SH3 and SH4, were selected as homozygous for the Sr33 transgene by screening with Sr33 sequence-specific primers.

**Functional testing of transgenic barley with Pgt races MCCFC and TKTTF**

To identify a Pgt race which would be virulent on Golden Promise and avirulent on Sr33 and Sr45, we interrogated a panel of 151 Ae. tauschii accessions which had their NLR repertoire sequenced (Arora et al., 2019) by BLAST search with the Sr33, Sr45, Sr46 and SrTa1662 gene sequences (using a ≥99% identity and 100% query coverage cut-off). We identified one accession that appeared to contain only Sr33, and four accessions that appeared to contain only Sr45. These accessions were resistant to Pgt race MCCFC, a race which was previously shown to be virulent on Golden Promise (Arora et al., 2013; Kleinhofs et al., 2009), whereas 31 Ae. tauschii accessions which did not appear to contain any of the aforementioned Sr genes were predominately susceptible or intermediate in their response to MCCFC (Table S19). From this, we concluded that MCCFC is avirulent towards Sr33 and Sr45. We also chose MCCFC for screening the barley Sr22 transgenics since this gene was previously postulated to be effective against this race with an infection type (IT) of 1; (Rouse and Jin, 2011).

**Wheat rust inoculations and phenotypic evaluations**

For the stem rust inoculations, Sr barley (Sr22, Sr33d, Sr35d, Sr45d) T1 or T2 plants alongside with the susceptible control cv. Golden Promise was infected with Pgt race MCCFC or/and TKTTF 10 days after planting. The inoculated plants were rated for disease response 12–14 days after inoculation as previously described in Yu et al. (2017). For Sr33, T1 plants alongside with the susceptible control cv. Golden Promise was infected with Pgt race MCCFC (isolate 59KS19) and TKTTF (isolate 13ETH18-1). Inoculation, incubation and disease assessment procedures were performed as described previously (Zhang et al., 2017). At least 10 plants from each homozygous family were evaluated for Sr33 and ITS were recorded once, 12 days after inoculation.

For the leaf rust experiment, each cone rack (98 cones × three seeds/cone = 294 plants/cone rack) received 1 mL of inoculum (15 mg spore) of P. hordei race 4 (Levine and Cherewick, 1952) across the primary leaves of 8–9 day-old seedlings. P. hordei isolate 12TX15-2 was used to inoculate the Sr33-transformed lines. Therefore, each plant received 0.05 mg of urediniospores. To minimise risk of phytotoxicity, the Soltrol 170 oil carrier was evaporated from the leaf surfaces by two hours of gentle fanning under 400-Watt HPS light bulbs. Inoculated seedlings were incubated at 22 °C inside mist chambers with 100% relative air humidity provided by a household ultrasonic humidifier for 18 h. Post inoculation, plants were moved to a greenhouse running a 16-hour day length with a night temperature of 15 °C and a day temperature of 20 °C. The resistant control used in the barley leaf rust infection assays was PI584760, which carries the gene Rph14 (Martin et al., 2020).

**Agronomic trait measurement**

Segregating T1 progeny derived from single copy (hemizygous) plants were sown in a glasshouse fitted with Heliospectra LED lights positioned to give a light intensity of 303 ± 68 μmol/m²/s at bench height and 467 ± 133 μmol/m²/s at canopy height (Table S20) and set to a 22 h light, 2 h dark growth ‘speed breeding’ regime as previously described (Ghosh et al., 2018). The leaves of three-week old plants were sampled for DNA extraction and qPCR (Bartlett et al. 2008) to distinguish between homozygous transgenic, hemizygous and nulls. The homozygous and null plants were potted up for further analysis. Tiller number (Figure 4e and Table S13), thousand grain weight (Figure 5; Table S14) and timing of progression to various growth stages including 3-leaf stage, node development, awn-peep, spike emergence, anthesis or grain dough stage (Figure S2; Tables S15-S18) was measured. A Welch’s t-test was conducted to determine if there were any significant differences between transgensics and nulls.

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**Conflicts of interest statement**

The authors declare no conflict of interest.

**Author contributions**

MAMdH, MS, RM, GY, NP and MA designed and generated Sr22, Sr33d, Sr35d and Sr45d constructs. MAMdH, MA and WH performed Sr22, Sr33d, Sr35d and Sr45d transformation. OM, MR and BJS phenotyped Sr22, Sr33d, Sr35d and Sr45d transgenics. SC, DB, XX, TR, RM and MA generated the Sr33 construct, performed transformation and selected homozygous lines while...
MNR phenotyped the transgensics. SA and BS performed sequence analysis. 5G and SA selected homoygous lines and performed experiments to capture growth and agronomic traits. BBHW, SKF, BJS, EL, NP and WH conceived and designed study. MAMM drafted manuscript with input from BBHW, BJS, MA, SP and NP. All authors read and approved the final manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1** Leaf rust infection assays with *P. hordei* race 4 on *Sr22, Sr33, Sr33d, Sr35d* and *Sr45d* representative *T*1 and *T*2 transgenics at the seedling stage.

**Figure S2** Timeline of growth stages (expressed as Days After Sowing) for *Hordeum vulgare* cv. Golden Promise with and without the presence of (a) *Sr22* (b) *Sr33d* (c) *Sr35d* (d) *Sr45d*. Boxplots indicate variation in timelines for the biological replicates. Suffixes ‘-N’ and ‘-H’ indicate nulls (for absence of the transgene) and homozygous (for presence of the transgene), respectively. Growth stages measured for the first tiller according to the Zadoks’ Scale (Zadoks et al., 1974).

Table S1 List of binary constructs carrying Sr gene.

Table S2 Stem rust infection assays with *Pgt* race MCCFC on *Sr22 T*2 homozygous lines.

Table S3 Stem rust infection assays with *Pgt* races MCCFC and TKTTF on *Sr33 T*2 homozygous lines.

Table S4 Stem rust infection assays with *Pgt* race MCCFC on *Sr33d T*2 homozygous lines.

Table S5 Stem rust infection assays with *Pgt* race TKTTF on *Sr33d T*1 homozygous lines.

Table S6 Stem rust infection assays with *Pgt* race MCCFC on *Sr45d T*2 homozygous lines.

Table S7 *Puccinia hordei* race 4 infection assays on *Sr22 T*2 families.

Table S8 *Puccinia hordei* race 4 infection assays on *Sr33 T*2 homozygous lines.

Table S9 *Puccinia hordei* race 4 infection assays with *Sr33d T*2 families.

Table S10 *Puccinia hordei* race 4 infection assays on *Sr33 T*2 families.

Table S11 *Puccinia hordei* race 4 infection assays on *Sr35d T*2 families.

Table S12 Stem rust infection assays with *Pgt* race MCCFC on *Sr35 T*1 families.

Table S13 Tiller number of *Hordeum vulgare* cv. Golden Promise with and without the presence of transgene.

Table S14 Thousand Grain Weight (TGW) of *Hordeum vulgare* cv. Golden Promise with and without the presence of transgene.

Table S15 Development stages of *Sr22* transgenics and nulls. Values indicated are expressed as days after sowing (DAS).

Table S16 Development stages of *Sr33d* transgenics and nulls. Values indicated are expressed as days after sowing (DAS).

Table S17 Development stages of *Sr35d* transgenics and nulls. Values indicated are expressed as days after sowing (DAS).

Table S18 Development stages of *Sr45d* transgenics and nulls. Values indicated are expressed as days after sowing (DAS).

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Table S19  Functional testing of Sr33 and Sr45 with Pgt race MCCFC.

Table S20  Photosynthetic photon flux density (PPFD) measurements for the LED-supplemented glasshouse. PPFD was measured in μmol/m²/s at a central location using an UPRTek MK350S spectrophotometer and associated uSpectrum software (UPRTek, Taiwan). Values are the mean of eighteen measurements ± the standard deviation taken in a metre square area under a light fixture. Plants were rotated around on a weekly basis.