Phenotypic and Molecular Characterisation of Silicon Uptake and Deposition in *Festuca arundinacea*

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Abstract

Silicon (Si) is taken up from the soil as monosilicilic acid by plant roots, transported to leaves and deposited as phytoliths, amorphous silica (SiO_2) bodies, which are a key component of anti-herbivore defense in grasses. Silicon transporters have been identified in important crop species such as rice and barley, but the mechanisms behind the transport of Si remain poorly characterised in many non-crop grasses. Specifically, the extent to which Si uptake and deposition is driven by Si transporter expression remains disputed. Induction of Si defenses in response to herbivory suggests plants exhibit control over Si uptake and distribution. This thesis investigated the effects of wounding and Si addition on foliar Si concentration and deposition, and on Si transporter gene expression, in different genotypes of the economically important forage grass *Festuca arundinacea*, which exhibits variation in Si uptake and deposition. Following Si addition and damage, varieties differed in Si concentration, the numbers of leaf spines, and in the magnitude of the increase in Si uptake induced by damage. Some previous studies suggest trade-offs may exist between Si and carbon, but thus far such potential trade-offs have not been investigated intra-specifically, nor have there been any tests of the existence of trade-offs between different types of Si-based defenses. Trade-offs between leaf spines and phytoliths, and between Si and the key structural component, lignin, were found. This thesis presents novel findings on how Si defenses are mobilised in response to damage, how they are regulated at the level of gene expression, and how Si is deposited in different structures on the leaf surface and within cells. These findings have implications for improved understanding of plant defense and for the targeted selection of traits during breeding for sustainable crop protection.

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Author's Declaration

I, Emma L. McLarnon, declare that this thesis is a presentation of original work and I am the sole author. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged as references.

Chapter 2 has been published as:

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Contribution to Hartley et al. 2015: The study carried out on *Festuca arundinacea* published within Hartley et al. (2015) was solely carried out by me (Emma L. McLarnon). This study was a pilot experiment to test the extent to which varieties were different in the Si they accumulated. The results of the experiment demonstrated the harsh variety accumulated more Si compared to the soft variety, and the way in which Si was deposited also varied between the two varieties. These results fitted well with results from other grass species being tested for similar differences in Si uptake and deposition, so were combined to produce a publication.

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1 Chapter 1: General Introduction

1.1 The importance of grasslands and forage grasses

Grasslands can be defined as "a habitat dominated by grasses" (Gibson, 2009), comprise ~26% of the world's total land area and 80% of agricultural land (Boval and Dixon, 2012), are the largest biomes on Earth (Gibson, 2009) and have important ecological and agricultural roles in terms of providing forage for both wild herbivores and livestock (Smit et al., 2008). Forage grasses provide feed for global domestic livestock production: extensive pastures provide 30% of total beef production and 23% for mutton (FAO, 1996). Savannas, grasslands, shrublands and deserts support a large proportion of managed pastoral systems (Asner et al., 2004) and these are all considered to be dryland biomes, which support 78% of global grazing (Asner et al., 2004).

The productivity and quality of grasses as forage for livestock is affected by a range of environmental factors. Similarly, the supply of nitrogen (N), phosphorous (P) and potassium (K) significantly influences both the composition of grasslands and the forage quality of plants found here: N, P and K fertilisation increases the dry matter yield and forage value of grasslands (Hejcman et al., 2007). The level of N is an important driver of forage quality and hence livestock production. Vegetation that has a low carbon to nitrogen ratio (C: N ratio) and high amounts of protein is optimal for grazing animals (Beeri et al., 2007). The response of plant species to nutrient supply is strongly affected by other environmental factors (Pennings et al., 2005; Hejcman et al., 2007). However, nutrient content is not the only driver of forage digestibility. The plant cell wall is formed from a complex network of chemical structures such as hemicelluloses, proteins and phenolic acids (Buanafina et al., 2008), and is difficult for ruminants to digest due to the chemical linking of these structural components. The digestibility of cell walls directly impacts livestock performance and the quality of the beef and milk they produce.

The impacts of climate change and the increased demands for food production by a population predicted to exceed 9 billion by 2050 (Martin et al., 2012) at the same time as increasing its meat consumption, are leading to an increase in the utilisation of marginal land for agricultural production, particularly livestock grazing (Martin et al., 2012). Such marginal land often suffers from lower soil fertility and higher levels of other abiotic stresses, such as high soil salinity, forage grasses able to resist these stresses are required to mitigate the effects of climate change on livestock production. One factor which is critical in reducing the impacts of a range of

abiotic stresses, including drought and soil salinity, on grasses is silicon. This element has these beneficial effects, but it is also a major defense in grasses which reduces forage quality and digestibility, so affecting the performance and behaviour of livestock. This thesis investigates the physiological and molecular basis of variation in silicon uptake in grasses. Exploiting this variation could be a way to select for forage grasses better able to thrive in saline or arid environments, or ones which are more digestible to livestock.

1.2 The importance of silicon (Si) for grasses: its role and uptake

Despite being classified as a non-essential element (Epstein, 1999), Si has been demonstrated to have many functional roles in plants (Cooke et al., 2016). One of the main functions of Si in grasses is defense against attack, namely protection against pathogens (Samuels et al., 1993), through surface deposition (Fauteux et al., 2005) as well as in terms of priming the production of plant defenses specifically involved in fighting pathogen and fungal attack, such as chitinases (Dann and Muir, 2002), and in protection against herbivores (Massey and Hartley, 2006; Massey et al., 2007b), where the physical deposition impedes access to nutrients (Hunt et al., 2008), abrades the gut of small herbivores (Wieczorek et al., 2015), and wears insect and vole mouthparts (Massey and Hartley, 2009; Calandra et al., 2016). As well as protection from biotic stresses, Si is also able to alleviate abiotic stresses (Cooke and Leishman, 2016; Manivannan and Ahn, 2017) such as heavy metal accumulation (Neumann and zur Nieden, 2001) and increased salt and drought tolerance (Yin et al., 2016; Khattab et al., 2014). In addition to increased stress tolerance and protection, Si is also involved in reinforcing the structure of the cell wall (Raven, 1983), where it can be found within the cell wall matrix.

Silicon is a metalloid (Pommerrenig et al., 2015) solubilised in the soil to form orthosilicic acid (H_4SiO_4). Plants readily take up Si when it occurs at pH <9 (Bauer et al., 2011) and it is then deposited within the plant in a range of forms, including solid, amorphous Si bodies (SiO_2) known as phytoliths (Piperno, 1988) produced by condensation of monosilicic acid.

Plants transport Si from the soil to inside the root cells by passive transportation mediated by an influx channel, Lsi1 (Figure 1.1.1). Lsi1 belongs to the aquaporin family of proteins (Ma et al., 2006; Bauer et al., 2011; Deshmukh and Bélanger, 2016). There are five main groups of aquaporins: plasma membrane intrinsic proteins (PIPs), Nodulin-like 26 intrinsic proteins (NIPs), tonoplast intrinsic proteins (TIPs), small basic intrinsic proteins (SIPs) and uncharacterised intrinsic proteins (XIPs) (Deshmukh and Bélanger, 2016). Lsi1 is a NIP and facilitates passive diffusion of small, uncharged molecules across the plasma membrane (Ma et al., 2006; Pommerrenig et al., 2015). Homologues of Lsi1 have been identified in a number of important crop species (Table 1.1) and continue to be identified as reduced sequencing costs and improved technology speed up the processes involved. Further, Lsi1 has two motifs (Asn-Pro-

Ala) (NPA) and six transmembrane domains which are the main characteristics of aquaporins (Ma et al., 2006). A recent study investigating the role of Casparian strips in Si transport found that both location and number of Casparian strips were important for Si accumulation, and may explain why rice accumulates so much Si, as it has two strips compared to other crop grasses which are reported to have only one (Sakurai et al., 2015).

Table 1.1: Cellular localisation of root influx and efflux Si transporters

Plant	Transporter	Cellular localisation	Reference
Rice	OsLsi1	Distal side of endodermal and exodermal cells of plasma membrane	Ma et al. 2006
	OsLsi2	Proximal side of endodermal and exodermal cells of plasma membrane	Ma et al. 2007
Barley	HvLsi1	Epidermal, hypodermal and cortical cells	Chiba et al. 2009
	HvLsi2	Root endodermis	Mitani et al. 2009b
Maize	ZmLsi1	Distal side of epidermal, hypodermal and cortical cells	Mitani et al. 2009a
	ZmLsi2	Root endodermis	Mitani et al. 2009b
Cucumber	CsLsi1	Distal side of endodermal and cortical cells	Sun et al. 2016
	CsLsi2	Plasma membrane	Wang et al. 205
Pumpkin	CmLsi1	All root cells	Mitani et al. 2011
	CmLsi2	Not known	Mitani-Ueno et al. 2011
Wheat	TaLsi1	Endodermal and cortical root cells	Monpetit et al. 2012
	TaLsi2	Not known	

The second root transporter, Lsi2, is not part of the aquaporin family, instead its characterisation and function are less well studied and understood than Lsi1. Lsi2 is found at the opposite polarity to Lsi1 in rice, suggesting that it is coupled with Lsi1 to carry out efficient transport of Si (Ma et al., 2007a). Lsi2 shares a motif with a gene *ArsB*, found in *Escherchia coli*, which encodes a protein for an efflux transporter of arsenic, and is inhibited by low temperatures and protonophores which show it is driven by a proton gradient (thus is energy-dependent), and therefore it is likely that Lsi2 is also an efflux transporter (Ma et al., 2007a). Lsi2 transporters have been identified in fewer species than Lsi1, but Lsi2 has 9-12 transmembrane domains which is the main distinguishing characteristic (Deshmukh and Bélanger, 2016). It is also thought that Lsi1 and Lsi2 are co-regulated as the promoter regions in rice *Lsi1* and *Lsi2* contain similar sequences of 150bp long (Yamaji and Ma, 2011).

Once the Si is transported by Lsi1 and Lsi2 to the xylem, it follows the flow of water to the shoot (Figure 1.1.1; Yamaji et al., 2008). In the shoot, two further transporters are involved in Si distribution: Lsi6 and Lsi3. Shoot Si transporters have received far less attention than root ones to date, though it is known that Lsi6 is a homologue of Lsi1 and therefore is a NIP. Lsi6 has similar properties to Lsi1 (2 NPA motifs), but it is expressed in the leaf sheaths and leaf blades (Figure 1.1.1; Yamaji et al., 2008). Lsi6 is found in the root tips also, and in rice the expression decreases with distance from root tips (Yamaji et al., 2008), suggesting Lsi6 is involved in detecting Si from the soil. In the shoot, Lsi6 is located in the xylem parenchyma in the leaf sheath and leaf blade, where its role is to unload Si from the xylem (Yamaji et al., 2008). Lsi3 shares 80% sequence identity with Lsi2 and has recently been characterised as a plasma membrane efflux transporter of Si (Yamaji et al., 2015). *Lsi6, Lsi2 and Lsi3* genes showed high expression in the node. *Lsi2 and Lsi3* were also found to be expressed in lower nodes and *Lsi3* showed expression in the peduncle and rachis (part of the inflorescence), whereas *Lsi2* was not expressed in these regions (Yamaji et al., 2015). Therefore, the movement of Si in the aboveground tissues is complex, and requires the efficient linking of these Si transporters to work together in order to move Si to areas of low transpiration (Yamaji et al., 2015).

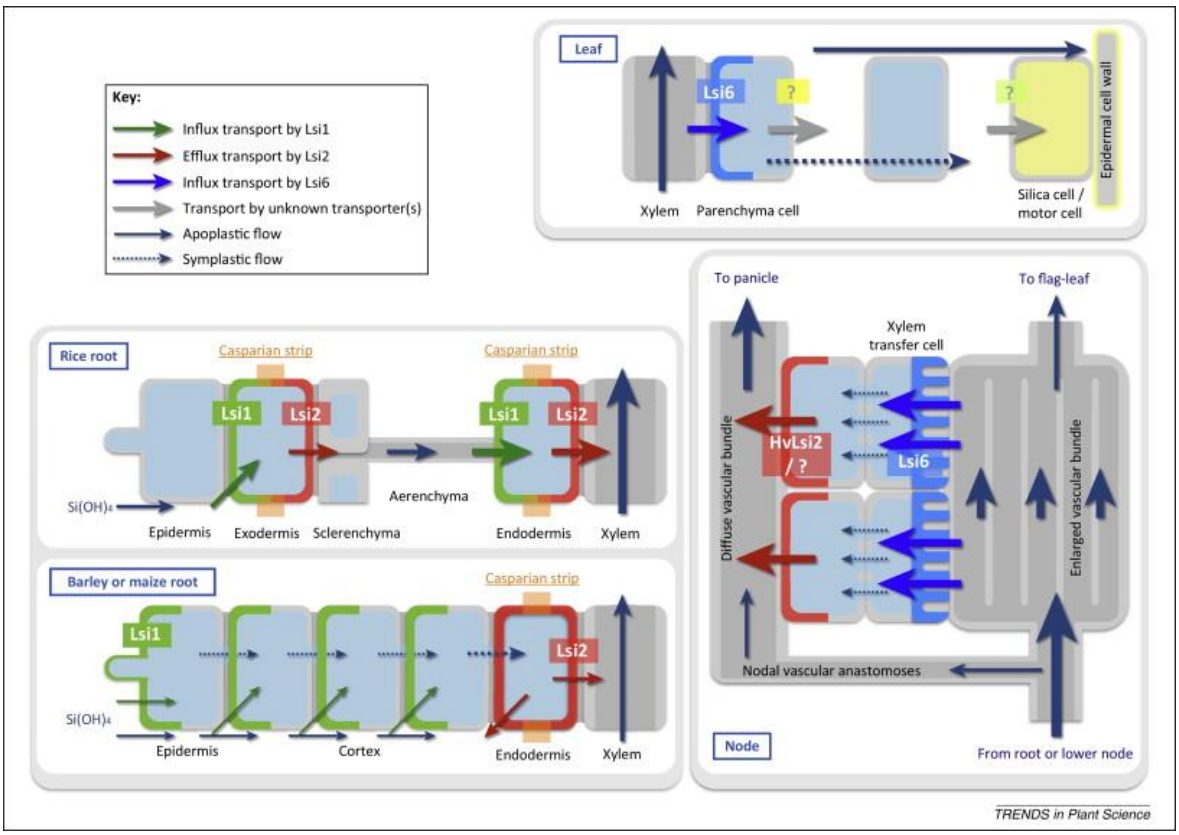

Figure 1.1.1: Schematic of Si transport from root cells to leaf cells. Diagram from Yamaji et al. (2015).

1.3 Silicon-based defenses

Silicon-based defenses are variable in form, with Si being deposited as phytoliths, within trichomes and various other cells within the plant (Piperno, 2006; Strömberg et al., 2016). Phytoliths are morphologically diverse both intra and interspecifically (Piperno, 1988; Figure 1.2). They also vary in their abrasiveness (Gügel et al., 2001) and thus their effectiveness of deterring herbivores. Abrasive, hard phytoliths on the leaf surface are thought to erode both invertebrate (Massey and Hartley, 2009) and vertebrate mouthparts (Jernvall and Fortelius, 2002; Rabenold and Pearson, 2011; Erickson, 2014). Recent studies have shown that plants with more abrasive phytoliths are more successful at defending their foliage from herbivory, compared with those which have lower levels of Si within the leaves and less abrasive phytoliths (Calandra et al., 2016), and that it is the number and type of these abrasive structures that explains the negative impact on herbivores, rather than the absolute Si concentration (Massey and Hartley 2009; Hartley et al., 2015). It has been suggested that phytoliths evolved as an adaptation to grazing herbivores (Erickson, 2014) and evidence shows that they are effective in deterring small mammalian herbivores (Massey and Hartley, 2006; Cotterill et al., 2007; Wieczorek et al., 2015) and insect herbivores (Massey and Hartley, 2009). However, other studies show phytoliths alone do not deter larger mammalian herbivores such as sheep (Massey et al., 2009; Hartley and DeGabriel, 2016), but they may underpin a reduction in bite

rate and nutrient acquisition, so hindering productivity of these larger mammals (Massey et al., 2009; Strömberg et al., 2016). It has also been suggested that Si deposition interferes with the rumen microbial community and thus reduces digestion of forages (Harbers et al., 1981; Mayland and Shewmaker, 2001; Agbagla-Dohnani et al., 2003).

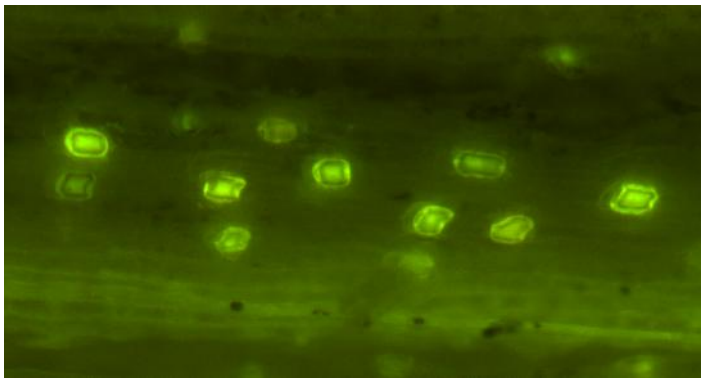

Figure 1.2: Spodogram (ashed plant material) of short-cell phytoliths in tall fescue. Green = Si deposition. Circular shaped objects = Silica short cells.

Grasses can induce Si-based defenses in response to repeated herbivory by increasing the amount of Si taken up from the environment (McNaughton and Tarrants, 1983; Massey and Hartley, 2006; Massey et al., 2007a, b; Massey et al., 2009). The exact mechanisms for induced Si-based defenses remain poorly characterised. There are currently two theories proposed to explain the increase in Si levels post-herbivory: the first theory suggests this is primarily due to Si translocation via the transpiration stream (Yamaji and Ma, 2014), where Si is deposited at sites of high water loss (Piperno, 1988; Exley, 2015). However, this does not explain observed differences in Si concentrations between different parts of the shoot. For example, the Si concentration of the flag leaf in rice is lower than that of the panicle and the node, which have lower transpiration rates than the flag leaf (Yamaji and Ma, 2014). These findings support the second theory, that Si uptake involves both passive and active elements (i.e. mediated by both transpiration and Si transporters), such that when Si reaches the shoot, it is redirected from the transpiration stream to areas of low transpiration via the active transporters Lsi2 and Lsi3 (Yamaji et al., 2015). Further to this, molecular evidence of inducible Si-based defenses have been reported: Ye et al. (2013) reported gene expression of the *Lsi1*, *Lsi2* and *Lsi6* increased in response to insect herbivory when additional Si was supplied; no significant gene expression differences were observed in plants in low Si environments. Further studies into the role of expression of the Si transporters in inducible based Si-defenses are required in order to fully understand the mechanisms underpinning Si uptake from a molecular perspective.

Further, induced Si-based defenses are not only found in response to plant herbivory; there is also evidence of increases in Si concentration after pathogen and fungal infections (van

Bockhaven et al., 2013; Vivancos et al., 2015; van Bockhaven et al., 2015; Whan et al., 2016; Manivannan and Ahn, 2017). It seems unlikely that increased transpiration can be responsible for the increased concentrations of Si after plants succumb to these infections. It is more likely that either by increasing Si transporter gene expression, the Si accumulation acts as a physical barrier to prevent further fungal penetration (Vivancos et al., 2015), or that increasing Si concentrations prime the defenses involved in plant protection (Fauteux et al., 2005; Chain et al., 2009). It is also thought that Si may interact with effector proteins involved in protection from pathogen attack (Vivancos et al., 2015). A number of recent studies have found that genes involved in plant defenses were increased in the presence of Si supply (Liang et al., 2003; Cai et al., 2008; Ye et al., 2013; Rahman et al., 2015; Reynolds et al., 2016). Ye et al. (2013) found that when the jasmonate pathway was stimulated in rice, Si accumulation was also increased, suggesting these defenses are linked. More work has been carried out into the effects of Si in priming of anti-pathogen defenses than anti-herbivore ones (Reynolds et al., 2016) and remains an area that requires further study.

1.4 Silicon uptake and other plant defenses

The relationship of how Si interacts with the structural elements (such as cellulose and lignin) of plants has been studied, but these studies are few in number. It has been suggested that Si may be used structurally in place of lignin, as it is more cost-effective due to reports of Si using 27 times less glucose to be incorporated into the cell wall than C (Raven, 1983; Cooke and Leishman, 2011b; Strömberg et al., 2016). There is also evidence of trade-offs between Si and C (Schoelynck et al., 2010; Cooke and Leishman, 2012; Strömberg et al., 2016), supported by the existence of negative relationships between Si and C (Ryalls et al., 2017) and between Si and C-based defenses such as phenolics (Frew et al., 2016). Cooke and Leishman (2011b) found that Si concentrations were higher in leaves of short-lived, non-woody plants, regardless of species, and this relationship extended to annual vs. perennial grasses, which supported their hypothesis that Si acts as a substitute for C when leaves are short-lived. This evidence supports the emerging theory that Si uptake and accumulation comes at a cost to the plant, due to the negative correlation between Si and leaf longevity (Cooke and Leishman, 2011b), although these costs remain to be fully understood, evidence from recent studies provides more support for this trade-off (Simpson et al., 2017).

The uptake of Si into the plant is mainly passive (i.e. does not expend energy), via aquaporins, but Lsi2 relies on a proton pump, which utilises energy (Ma et al., 2007a) and therefore there may be a cost involved in Si uptake, reflected in the trade-off between the types of defense employed by the plant. Understanding the relationship between other C-based leaf structural

traits (such as lignin and cellulose), and how they interact with Si is important in the context of forage grasses. Digestibility is important in forage selection and livestock performance, so there is a need to understand how Si will impact on digestibility traits that compose the cell wall. Ferulic acid (a phenolic acid found in the cell wall matrix) and diferulic acid make links with lignin in the cell wall and some studies have found that Si is able to link with ferulic acid (Buanafina and Fescemyer, 2012). It is these links between components in the cell wall that are difficult to digest in the rumen. Therefore, improving our understanding of these interactions between Si and other leaf structural traits is important in order to breed more palatable, digestible forage grasses.

1.5 Tall fescue as a study species

The *Festuca* genus in the Poaceae (grass) family comprises more than 600 species (Cheng et al., 2016). *Festuca arundinacea* Schreb. (tall fescue) is a cool season C_3, perennial grass (Gibson and Newman, 2001). C_3 grasses produce forages of higher nutritional quality that are more digestible compared to C_4 grasses, due to lower levels of fibre and lignin in C_3 grasses (Kephart and Buxton, 1992). Although once thought of as a single species (Gibson and Newman, 2001), the taxonomy of this species has led to the description of *F. arundinacea* as a species complex (Ekanayake et al., 2012) due to its three distinctive morphotypes: Continental - mainly found in Northern Europe; Mediterranean – found in Northern Africa; and Rhizomatous – found in Portugal and Spain (Hand et al., 2012b). It is a dominant pasture and turf grass in North America, Australia and Europe (Hand et al., 2012b). The Continental morphotype contributes to most temperate cultivars (Hand et al., 2010; Hand et al., 2012b; Figure 1.3for global distribution). Mediterranean tall fescue is found in Northern Africa and has greater winter growth, though lacks winter hardiness, compared to the Continental type (Hand et al., 2010; Hand et al., 2012b). Rhizomatous tall fescue is mainly utilised as a turf, due to its spreading ability aided by its prevalent rhizomes (Hand et al., 2012b).

Tall fescue is an outbreeding allohexaploid (contains six sets of chromosomes, derived from different species) (Hand et al. 2012a), though the ploidy level can vary between subspecies e.g. *F. arundinacea* subsp. *atlantigena* -St.Yves- Auquier is octaploid and *F. arundinacea* subsp. *fenas* Lag. -Arcang. is tetraploid (Cheng et al., 2016). Taxa belonging to this species complex have high levels of genetic diversity, owing to this range of ploidy (Cuyeu et al., 2013). This range of diversity means molecular studies on this species are, for the large part, lacking due to the difficulty in sequencing such a complex, large genome (5.27 – 5.83 gigabases (Gb)) (Lou et al., 2015); to put the genome size in context, rice (*Oryza sativa* L.) has the smallest cereal crop genome (430 megabases (Mb)) (Eckardt, 2000) and to date, barley (*Hordeum vulgare* L.) has one of the largest plant genome sizes (5.1Gb) (International Barley Genome Sequencing

Consortium, 2012). Currently no genome has been published for tall fescue and of the few studies that have attempted to increase the molecular knowledge, these have focussed mostly on the phylogeny of the species complex (Hand et al., 2010; Hand et al., 2012a,b; Cuyeu et al., 2013).

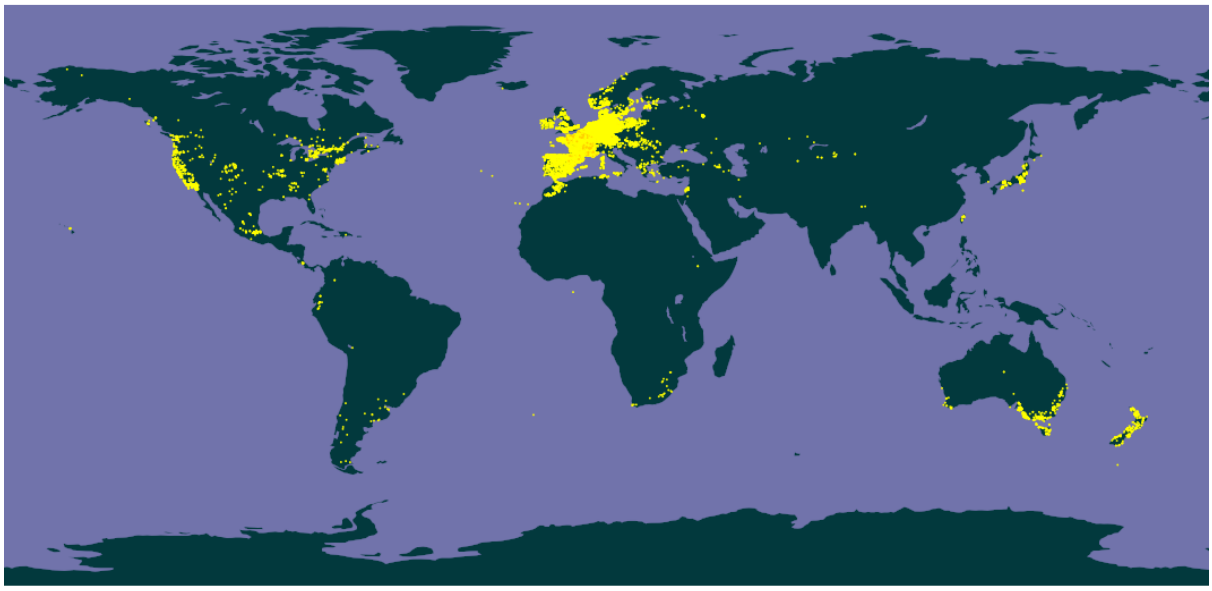

Figure 1.3: *Festuca arundinacea* global distribution (Map source: GBIF: https://demo.gbif.org/species/27062320). Yellow dots represent records of distribution.

Tall fescue was chosen as it is a highly important forage crop in livestock production, being widely grown for the beef and milk industries (Cuyeu et al., 2013). Tall fescue has been reported to be high yielding (Raeside et al., 2012; Cougnon et al., 2014), but it also has high lignin concentrations reducing its nutritive value relative to more nutritious forage grasses such as *Lolium perenne* (Pontes et al., 2007). However, tall fescue is more resilient to environmental stresses: when grown in Australia's high rainfall zones, it was able to persist in these adverse conditions, whereas *L. perenne* is known to suffer from poor persistence and low productivity (Raeside et al., 2012).

Although tall fescue has a wealth of attractive agronomic attributes, tall fescue is known for its rough leaf surfaces (Lou et al., 2015), potentially explaining why in mixed culture fields, cattle avoid tall fescue (Gibson and Newman, 2001), but the basis for this surface texture remains unknown. Several new varieties of tall fescue have been developed by plant breeders which are described as soft and more palatable to livestock (Keathley and Potter, 2011). Leaf texture therefore varies substantially between varieties but the reason for this has not yet been tested

experimentally. DLF Seeds Ltd provided a number of breeding varieties, currently involved in plant breeding trials, to investigate the relationship between leaf texture and Si deposition (Table 1.2). It has also been suggested that trichomes contribute to palatability issues with this species, as it has a leaf margin covered in trichomes (Gibson and Newman, 2001; Keathley and Potter, 2011).

Tall fescue accumulates Si in varying quantities (Ma, 2004; Hodson et al., 2005) – previous results from studies described above have shown a great deal of intertaxa variation in terms of the Si concentrations within the leaf. As an allohexaploid, *F. arundinacea* is comprised of multiple genomes, which suggests this may be in part responsible for the variation observed within the species in terms of the Si concentrations; it falls into both accumulator and non-accumulator categories (Ma et al., 2001; Hodson et al., 2005). Silicon uptake is plastic amongst grasses and varies depending on both species and environment (Hodson et al., 2005), but the heritability of genes relating to Si uptake in this species is unknown. The coarse leaf texture of tall fescue may be a result of Si accumulation and deposition within the leaf epidermis. As this trait (rough leaf texture) reduces forage quality and palatability, it is important to understand the mechanisms underpinning this trait.

Table 1.2: Varieties of *F. arundinacea* studied

Variety	Leaf texture	Chapter
DLF-01	Very very soft	2, 3, 4
DLF-02	Very soft	3, 4
DLF-03	Soft	2, 3
DLF-04-D	Soft	5
DLF-05-J	Soft	5
DLF-06-8	Soft	5
DLF-07-F	Soft	5
DLF-08	Semi-soft	3
DLF-09	Semi-harsh	3
DLF-10-M	Harsh	2, 5
DLF-11-C	Harsh	3, 4
DLF-12-T	Harsh	5
DLF-13-K	Harsh	5
DLF-14-N	Harsh	5

1.6 Aims and objectives

The aim of this thesis is to understand and elucidate the underlying mechanisms involved in the nature of inducible Si-based defenses, both in terms of uptake of Si and also deposition of Si into different Si-based defensive structures, such as phytoliths and trichomes. Tall fescue is reported to have rough leaf surfaces, the extent of which varies between genotypes, yet the basis for this trait remains unknown. The working hypothesis is that the genotypes with rough leaf surfaces have more Si deposited. Further, if this is the case, then understanding how these different leaf textures are achieved is necessary, i.e. are more trichomes or phytoliths deposited in the genotypes with harsh leaf surfaces? There have been relatively few studies which have investigated genotypic differences in Si uptake and deposition; understanding trends relating to Si uptake and deposition at the intraspecific level will give a clearer idea about the heritability of Si uptake, and the influence of genotype on Si acquisition. These genotypes will also provide model systems to investigate inducible Si-based defenses within this species. If the harsh varieties accumulate more Si and deposit more Si, will these varieties respond more efficiently to damage and Si supply by increasing Si uptake, and which structures are silicified under these conditions? Further, are these differences in Si uptake mediated solely by transpiration, or is there a molecular element to this induction of Si in terms of Si transporter gene expression? Finally, Si and C trade-offs have been found in other species, is there a trade-off between Si and C in these genotypes and how does this affect other leaf structural traits such as lignin concentration?

The main aim of this work was to understand the relationship between Si and leaf texture in a number of breeding varieties of tall fescue, in an attempt to understand how Si is associated with issues surrounding palatability. The objective of this work is to improve understanding of traits that can be used for targeted selection for improved palatability. A range of varieties described over a scale of “very very soft” up to “harsh” were provided for study by the grass breeding company DLF Seeds Ltd. (Table 1.2).

The main hypotheses of the research are:

1. Harshness is related to Si uptake and deposition, therefore varieties described as harsh will accumulate more Si and deposit it in abrasive forms such as within trichomes. This deposition will contribute to the harshness associated with the leaf texture.
2. Plants that accumulate more Si in response to damage do so by actively increasing Si uptake, reflected in the higher gene expression of Si transporters.

3. As there is a cost involved in Si uptake by Lsi2, this will be offset by reducing C-based structures involved in the cell wall, and therefore there will be a negative relationship between Si and C-based leaf structural traits.

Each chapter addresses questions which have not been studied or where only few studies exist:

Chapter 2: To understand how Si contributes to leaf texture in *F. arundinacea*. It is known that tall fescue accumulates Si, but the range of this is limited in the literature (Hodson et al., 2005). Using a range of varieties differing in leaf texture, this thesis aims to understand to what extent Si concentration contributes to leaf texture within this species.

Chapter 3: The deposition of Si occurs in various structures, suggesting that the way in which Si is deposited has an influence on palatability. The aim is to characterise and quantify the silicified structures in the different breeding varieties, and to understand how Si concentration is related to the Si-based structures deposited on the leaf. Trade-offs between Si and C have been found in some previous studies. This work aims to understand if such trade-offs occur within tall fescue varieties between Si and C-based leaf traits and/or between different Si-based structures (e.g. trichomes and phytoliths).

Chapters 4 and 5: The thesis also tests whether inducible Si-based defenses are passive, i.e. mediated solely by changes in transpiration, or if there is an active element to the increase in Si uptake in response to damage in tall fescue. The extent to which this active element to Si uptake, if it exists, is driven by changes in the expression of the genes encoding for Si transporters in the root was also tested.

2 Chapter 2: Response to silicon supply between two varieties of tall fescue

Chapter two comprises the published manuscript Hartley et al. 2015 (Defending the leaf surface: intra- and inter-specific differences in silicon deposition in grasses in response to damage and silicon supply), which is detailed in sections 2.10 -2.15. Supplementary analysis from the experiment relating to *Festuca arundinacea* described in Hartley et al. 2015 is detailed in sections 2.16 – 2.20 as separate hypotheses were tested, not relevant to the manuscript (see Author's Declaration for further detail).

2.10 Defending the leaf surface: intra- and inter-specific differences in silicon deposition in grasses in response to damage and silicon supply

2.11 Introduction

Grasslands including managed rangelands and pastures cover ~40% of the Earth's surface and grasses are an important plant family agriculturally, economically and ecologically (Strömberg, 2005; Gibson, 2009). Not only are our most widely grown and consumed food crops domesticated grass species, but grasses also provide grazing for both wild and domesticated animals. In their long co-evolution with grazers (Coughenour et al., 1985), grasses have developed a number of defensive strategies to both tolerate and repel herbivory (Vicari and Bazely, 1993), including rapid regrowth ability from their basal meristems (also an adaptation to fire and trampling common in these ecosystems) and a combination of both chemical defenses (including those provided by endophyte mutualists; Hartley and Gange, 2009) and physical defenses (McNaughton and Tarrants, 1983).

One such physical defense is the accumulation of silicon (Si) which has been previously reported to accumulate in high levels in the leaves of many grass species (Hodson et al., 2005), although the amount of Si accumulated shows large inter and intra species variation (Massey et al., 2007a; Soininen et al., 2013). There is clear evidence to demonstrate that these high levels of Si are effective anti-herbivore defenses, with impacts on the feeding preferences and performance of both vertebrate (McNaughton and Tarrants, 1983; Gali-Muhtasib et al., 1992; Massey and Hartley, 2006; Teaford et al., 2006; Massey et al., 2009) and invertebrate herbivores (Goussain et al., 2005; Massey et al., 2006; Kvedaras et al., 2007; Massey and Hartley, 2009).

These adverse effects appear to be mediated at least in part by abrasion: Si is primarily deposited as amorphous silica in the form of solid bodies known phytoliths in the epidermis (Richmond and Sussman, 2003; Currie and Perry, 2007). Phytoliths are hard and often irregular shapes and Si is also deposited in leaf hairs, trichomes and spines; all these structures could influence the texture and abrasiveness of the leaf. It has been suggested that Si abrades the teeth of mammalian herbivores (Jernvall and Fortelius, 2002; Erickson, 2014 but see Sanson et al., 2007) and an increase in leaf abrasiveness has been shown to reduce the performance of both vertebrate and invertebrate herbivores. For example, the amount of mandible wear feeding imposed on African armyworm (*Spodoptera exempta* Walker), and hence the reduction in their ability to extract nitrogen from their food, is correlated with the Si levels of the foliage they consume (Massey and Hartley, 2009), whilst voles prefer, and perform better on, grasses which are less abrasive (Massey and Hartley, 2006; Massey et al., 2008).

Previous work has suggested that foliar Si levels and the abrasiveness of grass leaves are reasonably well correlated: over 70% of the variation in abrasiveness across 18 different grass species was explained by Si content (Massey et al., 2007a). However, Si levels and abrasion are not always closely linked. For example, despite containing similar concentrations of Si, *Festuca ovina* L. was found to have much higher levels of abrasiveness compared to *F. rubra* L., whilst increasing leaf Si concentration through Si addition produced a smaller increase in abrasiveness in *Poa annua* L., a relatively palatable species, than in the more unpalatable *Brachypodium pinnatum* (L.) P. Beauv. (Massey et al., 2007a). It is possible that different grass species deposit their available Si differently at their leaf surfaces, influencing the abrasiveness of their leaves. It is certainly well-known that phytolith morphology varies between plant taxa, with differences between species sufficiently marked and consistent to allow phytoliths to be useful in palaeobotany (Strömberg, 2005). Some phytoliths are relatively smooth in shape, others much less so and it seems likely that the size, shape and density of phytoliths and Si rich spines will influence the abrasiveness of the leaf surface and its impact on the preferences and performance of herbivores.

Another influence on the nature and effectiveness of the leaf surface defenses will be the amount of Si available in the soil to take up and deposit (Currie and Perry, 2007). Previous exposure to herbivory has also been shown to impact on the levels of Si-based defenses in plants. It has long been known that Si levels increase in grasses from grazed areas (McNaughton and Tarrants, 1983) and herbivore-specific induction in Si defenses has been shown to occur, but only after repeated damage above a threshold (Massey et al., 2007b; Reynolds et al., 2012). More recently it has been shown that there are differences in both grass species and grass genotypes in the extent to which they respond to damage with increased Si uptake (Soininen et al., 2013).

The aim of this study was to determine the leaf Si concentration of different forage grass genotypes and naturally occurring grass species previously reported to differ in their leaf abrasiveness (see below), and to investigate whether these differences in leaf texture are related to the way Si is deposited on the leaf surface, potentially influencing the effectiveness of their use of Si in terms of reducing palatability to herbivores. We hypothesized that:

(i) harsher and more abrasive species and varieties would have higher leaf Si levels than softer ones;

(ii) species with similar Si levels which differed in abrasiveness would do so because they used their Si to produce a greater number of phytoliths and/or spines on their leaf surface, and that these spines would be larger or sharper.

We also hypothesized that irrespective of grass species, foliar Si levels would be elevated by increases in Si supply, and hence in potential uptake (Epstein, 1999; Cooke and Leishman, 2011a), and by damage, due to induction (Massey et al., 2007b). We also expected foliar Si levels to be highest in plants receiving both Si addition and damage, since induction in response to damage would be able to capitalize on the additional Si available in the soil. We predicted that the most abrasive species would deposit this additional Si in the form of surface spines to a greater extent than less abrasive species.

2.12 Materials and Methods

2.12.1 Study Species

Festuca arundinacea Schreb. is a cool season perennial grass (Gibson and Newman, 2001) and a dominant pasture and turf grass in North America, Australia and Europe (Hand et al., 2012b). It has a number of attractive agronomic attributes, including high yields, winter persistence (Gibson and Newman, 2001) and tolerance to drought (Cougnon et al., 2014), though it appears to be relatively unpalatable to cattle. In mixed culture fields, cattle rarely choose it as their forage choice (Gibson and Newman, 2001), possibly because of the “harsh” (i.e., feeling rough to the touch) texture of the leaf surfaces. There is interest amongst forage breeders in understanding the basis of this leaf harshness and unpalatability to improve the attractiveness of this species as forage. A number of varieties of *F. arundinacea* ranging from very harsh to very soft leaf textures have been developed by plant breeders (based on manual evaluation of surface roughness in the field by plant breeders), enabling the testing of the hypothesis that Si has a role in causing the harsh leaf surfaces.

We can also address the relationship between Si content and leaf texture by exploiting the natural variation in the relationship between Si and abrasion across native non-forage *Festuca*

species: *F. ovina* and *F. rubra* may differ so markedly in their leaf abrasion despite similar foliar Si levels (Massey et al., 2007a) because of the way they utilize the Si they take up. Specifically, *F. ovina* may produce a greater number, larger or more abrasive spines and phytoliths than *F. rubra*. We compared these species with the Si content and leaf texture of *Deschampsia cespitosa*, a grass known to be particularly unpalatable to herbivores due to its Si defenses (Massey and Hartley, 2006, 2009).

2.12.2 Plant Growth and Experimental Treatments

The two varieties of *Fesctuca arundinacea* were grown individually from seed in a loam based compost (John Innes No.2) in 13 cm pots. Both varieties were harvested 8 weeks after sowing at the point where all plants had at least four tillers. *F. ovina*, *F. rubra*, and *D. cespitosa* (L.) were grown from seed individually in peat based F2 (Levington, Scotts) compost in 10 cm diameter pots in the greenhouse conditions 16 h daylight, 20 °C/15 °C day/night. Due to their slower growth rates in relation to *F. arundinacea*, these three species were harvested 18 weeks after sowing.

Once established, plants were randomly assigned to four treatments: control plants with no Si addition and no damage (Si- D-), Si addition only (Si+ D-), damage only (Si- D+), and both Si addition and damage (Si+ D+). Treatments were imposed 3 weeks after sowing in the case of *F. arundinacea*, with plants harvested 5 weeks later, and 8 weeks after sowing in the case of the other three species, which were harvested 10 weeks later. There were six replicate plants of each treatment combination for *F. arundinacea* and seven replicate plants of each treatment for the other species.

For all grass species and varieties, Si addition was achieved by watering plants with 150 mg L^{-1} solution of dissolved sodium metasilicate ($Na_2SiO_3 \cdot 9H_2O$). Plants were watered 100 ml twice a week with either Si solution or deionised water. *F. ovina*, *F. rubra*, and *D. cespitosa* plants in the two treatments where damage was applied were mechanically damaged using scissors once a week over 10 weeks. Half of the plant's leaves were damaged by removing approximately half the leaf lamina down the midrib; the remaining leaves were left undamaged. When damaged plants were harvested, damaged and undamaged leaves were kept separated for Si analysis in order to test for induction of Si defenses in both the undamaged and the damaged leaves on the damaged plants.

2.12.3 Silicon Analysis by Portable X-Ray Fluorescence Spectrometry (P-XRF)

Silicon was analyzed by P-XRF, calibrated using Si-spiked synthetic methyl cellulose and validated using Certified Reference Materials of NCS ZC73014 'Tea' obtained from China National Analysis Center for Iron and Steel (Reidinger et al., 2012).

Both P-XRF and energy dispersive x-ray spectroscopy (see below) work on the principle of excitation of inner orbital electrons by an X-ray radiation source. As the excited electrons relax to the ground state, they fluoresce, thereby ejecting photons of energy and wavelength characteristic of the elements present and their concentrations. XRF instruments are widely used for the non-destructive, rapid and accurate elemental analysis of a range of materials (Jang, 2010).

Leaf material was ball milled (Retsch MM 400, Haan, Germany) for 2 min at a vibrational frequency of 24 Hz (60 min^{-1}) with two 1 cm diameter steel grinding balls in a 25 ml grinding jar. Leaf material was pressed at 11 tons for approximately 5 s into 5 mm thick cylindrical pellets with a manual hydraulic press using a 13 mm die (Specac, Orpington, UK). Silicon analysis (% Si DW) was performed using a commercial P-XRF instrument (Niton XL3t900 GOLDD analyser: Thermo Scientific Winchester, UK) held in a test stand (SmartStand, Thermo Scientific, Winchester, UK).

2.12.4 Surface Analysis by Scanning Electron Microscopy (SEM) and Energy Dispersive X-Ray Spectroscopy (EDX)

Leaf samples were taken from two replicate plants per species from all four treatment combinations for the inter-species experiment. A square section (~5 mm^2) of leaf material either side of the midrib of a mature, expanded leaf blade on the main stem was cut with a razor blade and immediately placed in fixative (2.5% glutaraldehyde, 4% formaldehyde in 100 mM phosphate buffer). For the *F. arundinacea* experiment, samples were taken from the harsh variety with added Si and the soft variety with no added Si (Si addition had no effect on Si levels in this experiment – see below). A square section (~1 cm^2) spanning the entire width of the mature, expanded leaf blade was cut from the main stem for each variety. The samples were then dehydrated through an acetone graduated series (samples were placed at 25, 50, 75, and 100% acetone concentration for ~1 h) and critical-point dried. Samples were then mounted on sticky carbon tabs and coated with 8nm thick layer of platinum-palladium.

SEM images were obtained using FEI Sirion S-FEG FESEM (Oxford Instruments, Tubney Woods, Abingdon, Oxfordshire). EDX was used in conjunction with the SEM to determine the elemental composition of the samples; an electron beam was focused on the samples and the difference between the ground state (unexcited state) and the excited state was measured by the energy-dispersive spectrometer which determines the elements present in the sample (Goldstein, 2003). The EDX analysis was performed using an Oxford INCA analysis system FESEM (Oxford Instruments, Tubney Woods, Abingdon, Oxfordshire), using the working distance of 10 mm. For the SEM images, the voltage was 5–10 kV and for the EDX analysis the voltage was 12 kV.

2.12.5 Statistical Analyses

All analyses were performed using R (version 3.0.2). ANOVA was used to test the main and interactive effects of grass species or genotype, Si addition and damage treatments on leaf Si concentrations. The effects of the Si and damage treatments were assessed on undamaged leaves from plants across all four treatments, to test if damage led to increased Si levels systemically in damaged plants in comparison with undamaged plants. A separate analysis tested for the effect of these treatments in damaged leaves from damaged plants compared to undamaged leaves from undamaged plants. *Post hoc* Tukey contrast tests were performed using the ghlt function from multcomp package (Hothorn et al., 2014).

Linear models were used to check for normality and homogeneity of variance following Crawley (2007). Silicon (%) values were transformed using the arcsine square root transformation to meet the assumptions of the test. Significance was set at $P < 0.05$ for all analyses.

2.13 Results

2.13.1 Intraspecific Differences

The *F. arundinacea* variety with a harsh leaf surface texture had significantly higher leaf Si concentration than the soft texture variety ($F_{1, 19} = 8.586$, $P < 0.01$), but there was no significant interaction between Si addition and variety ($F_{1, 19} = 0.282$, $P > 0.5$; Figure 2.1).

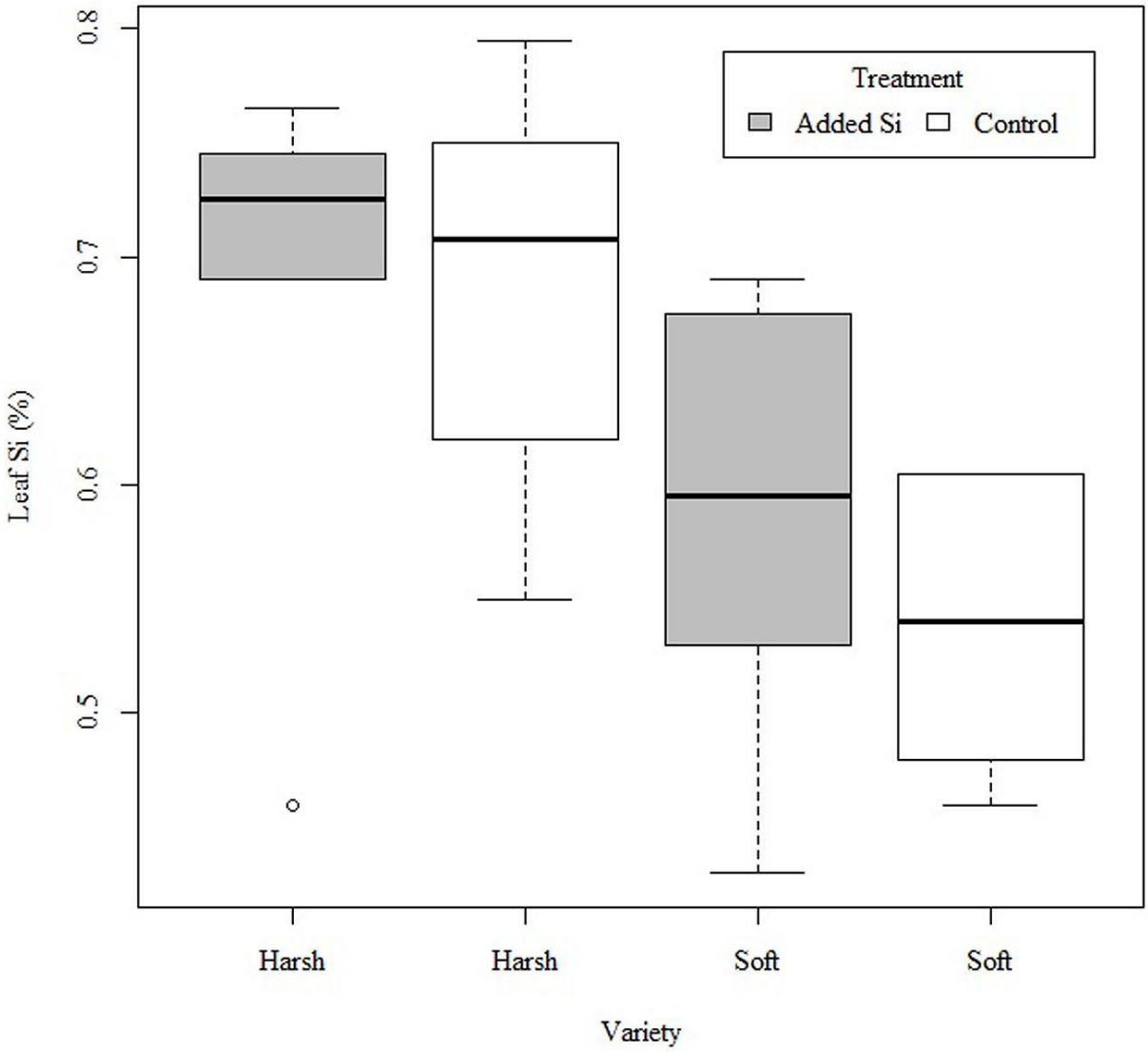

Figure 2.1: Silicon (Si) concentration (%) of harsh and soft variety of *Festuca arundinacea* with no Si addition (control) or Si addition (+Si).

The two main types of cells which were silicified were leaf spines (trichomes) and silica short cells. The harsh variety had more spines present on the abaxial surface than the soft variety (Figure 2.2A, B), which not only had fewer spines but the spines which were present were smaller and had a different morphology (Figure 2.2B, C). The spines present on the harsh variety were bigger in size and the point of the spines were spear-like in appearance; these spines also appeared to protrude more from the surface compared with the soft variety, where the spines were smaller in size and the points of the spines lay closer to the surface of the leaf. No spines were observed on the adaxial surface of either variety of *F. arundinacea* (images not shown).

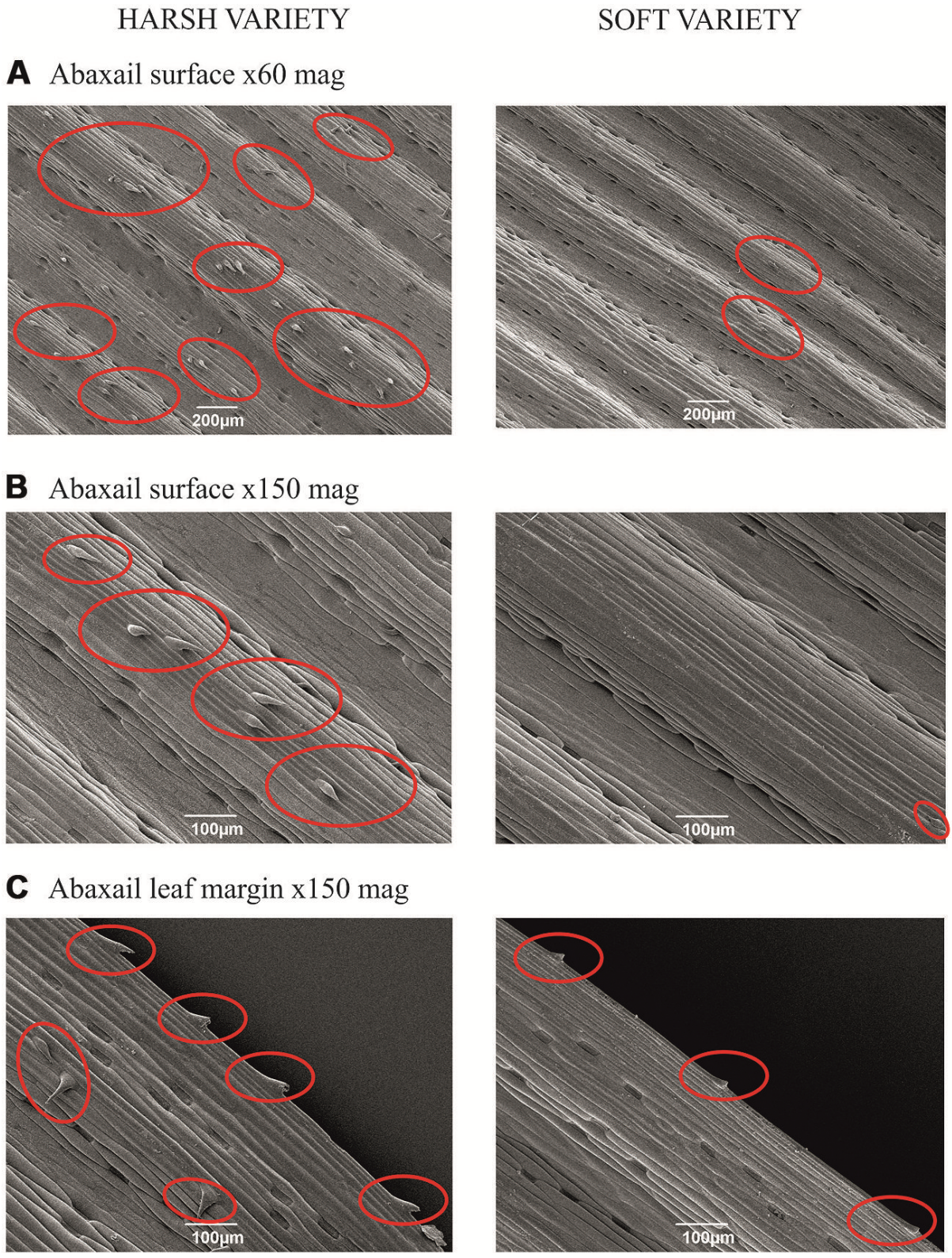

Figure 2.2: (A) Harsh and soft variety abaxail surface (x60 magnification), (B) Harsh and soft variety abaxail surface (x150 magnification), (C) Harsh and soft variety abaxail margin (x150 magnification). Red circles indicate leaf spine presence.

The spines were rich in Si (Figure 2.3), and there were other Si deposits on the leaf surface in the form of silica short cells. Generally, the harsh variety had a greater over surface deposition

of Si compared with the soft one, depositing the Si within the leaf spines (red arrows Figure 2.3), and also silica short cells surrounding the spines (red circles Figure 2.3). The soft variety deposited Si as silica short cells on the leaf surface within fewer, smaller leaf spines containing less Si than in the harsh variety (Figure 2.3B).

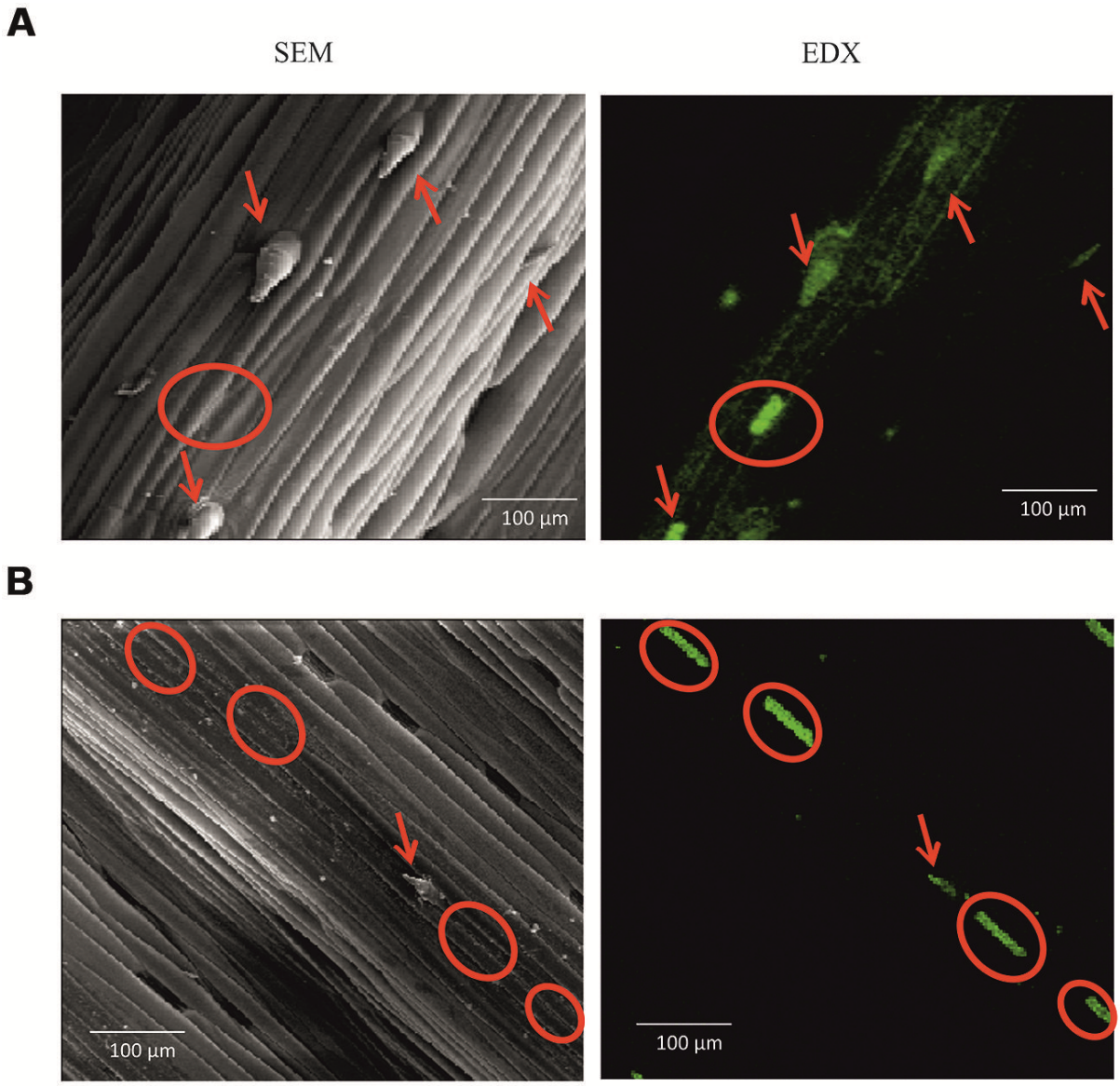

Figure 2.3: (A) Harsh variety abaxail surface (x200 magnification), (B) Soft variety abaxail surface (x200 magnification). Scanning electron microscope (SEM) represented by grey images. Electron density X-ray spectroscopy (EDX) represented by black and green images; green intensity indicates Si concentration. Red arrows indicate trichomes with Si deposition. Red circles indicate silica short cells.

2.13.2 Interspecific Differences

Grass species differed in their leaf Si concentration (Figure 2.4; Spp effect: $F_{2,72} = 23.62$, $P < 0.001$ undamaged leaves; $F_{2,72} = 15.99$, $P < 0.001$ damaged leaves) with significantly lower Si

concentrations in *D. cespitosa* compared to *F. rubra (post hoc* Tukey tests $P < 0.05$ for both undamaged and damaged leaves) and *F. ovina (post hoc* Tukey tests $P < 0.01$ for both undamaged and damaged leaves*)*. Plants treated with Si addition responded with an increase in their leaf Si concentration irrespective of whether leaves were damaged or not (Si effect $F_{1,72}$ = 1265.33, $P < 0.001$ undamaged leaves; $F_{1,72} = 463.17$, $P < 0.001$ damaged leaves). In comparison with undamaged leaves on undamaged plants, damage did not increase Si levels in undamaged leaves on damaged plants ($F_{1,72} = 0.03$, $P > 0.05$ NS), but there was a significant increase in the Si levels in the damaged leaves ($F_{1,72} = 17.92$, $P < 0.001$), suggesting that damage-induced increases in Si levels are localized in damaged leaves and do not spread to undamaged ones on the same plant (Figure 2.4).

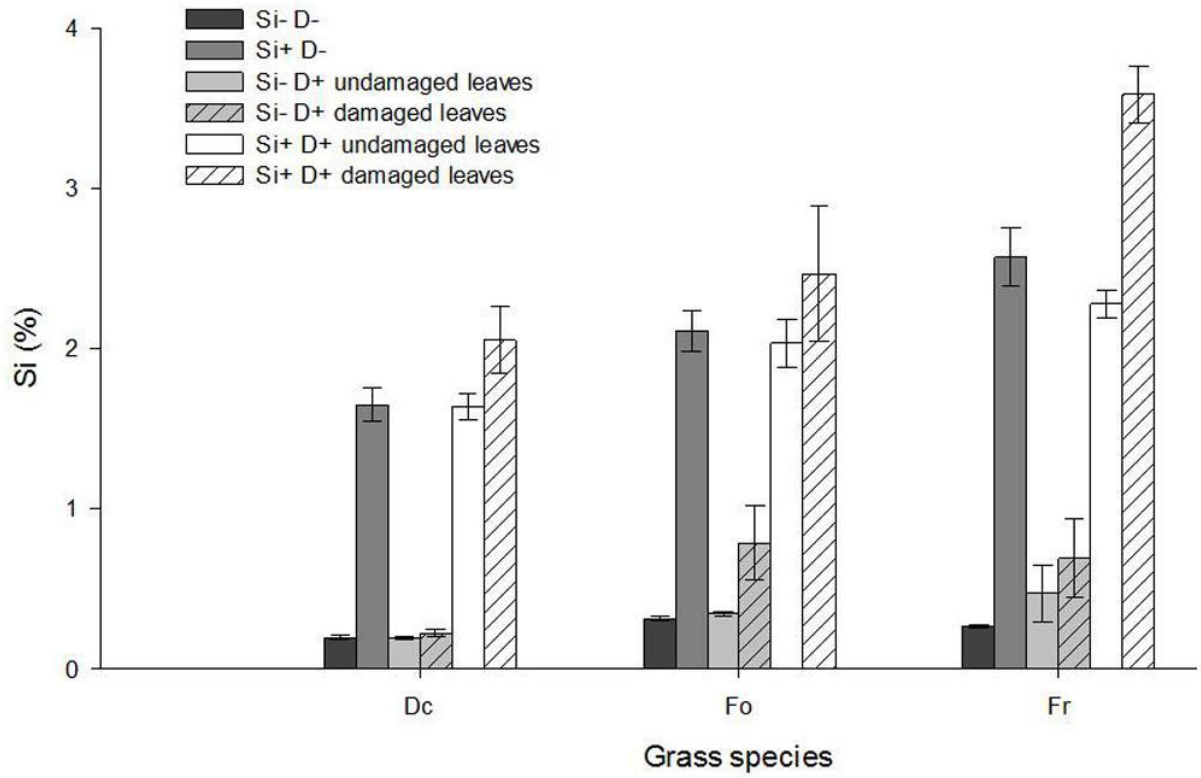

Figure 2.4: Leaf Si concentration (%) of *D. cespitosa* (Dc), *F. ovina* (Fo) and *F. rubra* (Fr) plants treated with no Si addition and no damage (Si- D-), Si addition only (Si+ D-), damage only (Si- D+), and both Si addition and damage (Si+ D+). Values represent mean ± SE bars of seven replicates.

In the case of undamaged leaves there was no significant interaction between the effects of species and Si addition on Si levels ($F_{2,72} = 2.89$, $P > 0.05$ NS), suggesting all three species responded in a similar way to increases in Si supply in terms of the allocation of this additional

Si to their undamaged leaves. However this was not the case for damaged leaves, where a significant Species × Si addition interaction ($F_{1,72} = 4.62$, $P < 0.05$) suggests species differ in where they allocate any additional Si once they are damaged. This is confirmed by the *post hoc* Tukey tests which showed that *F. rubra* had significantly higher concentrations of Si in damaged leaves under conditions of increased Si supply than either *F. ovina* ($P < 0.01$) or *D. cespitosa* ($P < 0.001$; Figure 2.4).

The SEM revealed differences in Si deposition on the leaf surfaces of the grass species (Figure 2.5). The leaf surface of *D. cespitosa* plants was found to have abundant Si-rich leaf spines (trichomes), even in the absence of Si addition. In contrast, the leaf surface of *F. rubra* and *F. ovina* plants growing without added Si had only rounded silica short cells and no leaf spines, although the round phytoliths were much more prominent and frequently distributed on the leaf surface of *F. ovina* than *F. rubra*. Both *F. ovina* and *D. cespitosa* plants deposited additional phytoliths (silica short cells) in response to increased Si supply, especially in the presence of damage, but Si addition had very little effect on the number or shape of the phytoliths deposited on the leaf surface of *F. rubra*. The damage alone treatments had little effect on leaf surface Si deposition in any of the grass species (images not shown).

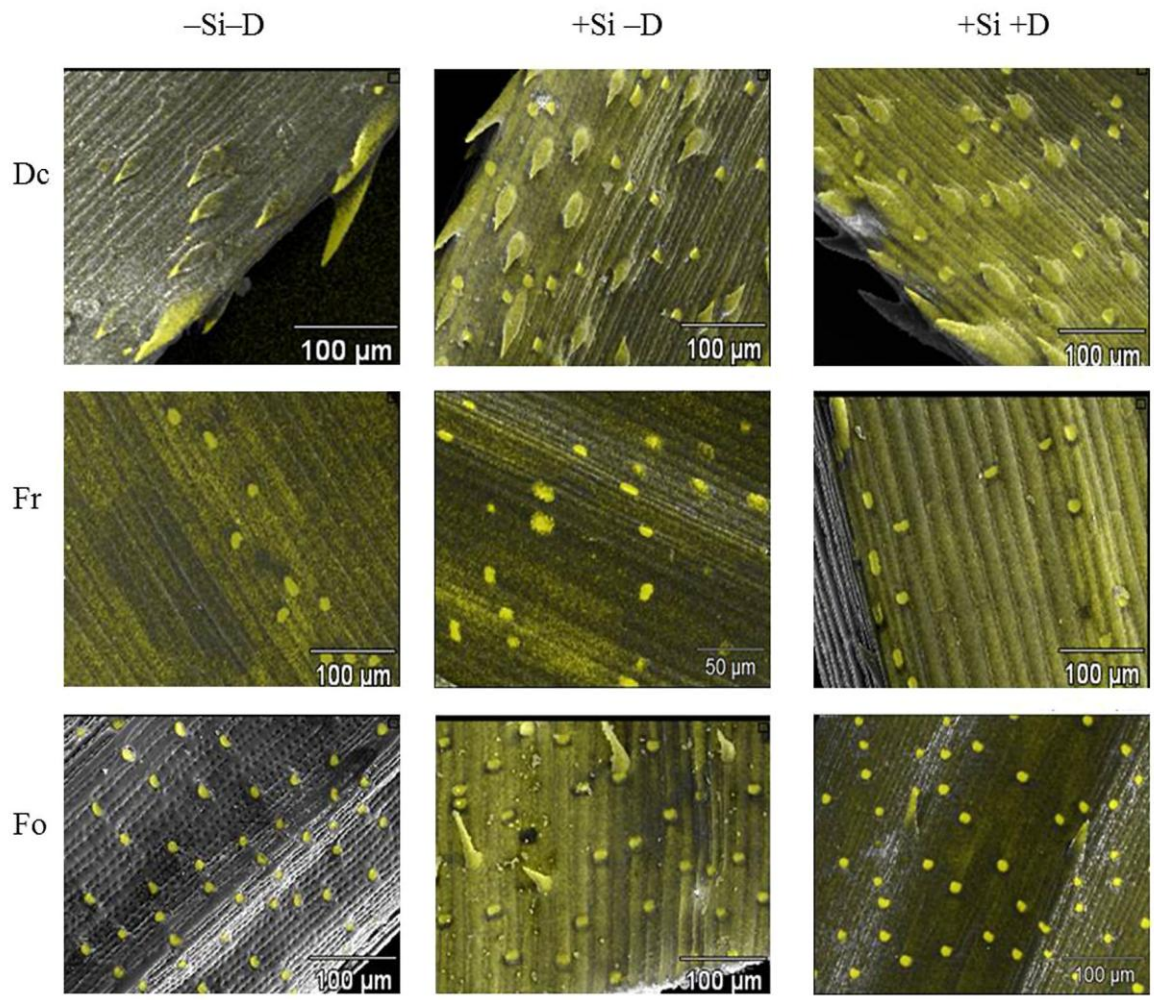

Figure 2.5: SEM-EDX images (x300 magnification) of *D. cespitosa* (Dc), *F. ovina* (Fo), and *F. rubra* (Fr) plants treated with control (Si- D-), Si addition with no damage (Si+ D-) and Si addition and damage (Si+ D+). Yellow intensity indicates Si concentration.

2.14 Discussion

Grass species and varieties differed in their leaf Si concentrations and the form in which this Si is deposited on the leaf surface. The more unpalatable and abrasive species, namely *D. cespitosa* and the harsh variety of *F. arundinacea* had both larger and a greater abundance of Si-rich spines compared to the more palatable *F. rubra* and soft variety of *F. arundinacea*. Silicon addition resulted in an increase in leaf Si concentration in three out of the four grass species and altered the deposition of Si on the leaf surface in the case of *D. cespitosa* and *F. ovina*, but had little impact on the surface of *F. rubra*. The different forms in which Si is deposited at the leaf surface in *F. ovina* and *F. rubra* may explain previous observations that they differ in abrasiveness even though, as we found here, they contain similar amounts of Si. Generally, damage caused a small increase in leaf Si concentration, but did not have a large effect on the form in which the Si was deposited on the leaf surface.

2.14.1 Intraspecific Differences

As we hypothesized, the harsh variety of *F. arundinacea* had a higher leaf Si level than the soft variety, though our prediction that Si addition would increase foliar Si content was not supported in this species. The differences in foliar Si content between the harsh and soft varieties suggest that Si is contributing significantly toward the differences in leaf texture between them. The harsh variety had significantly higher leaf Si content than the soft variety as well as having a different leaf surface morphology, suggesting that increased levels of Si support the production of increased number and size of leaf spines. This is significant in the context of forage grass: differences in the patterns of deposition of Si on the leaf surface between varieties may offer scope for plant breeders to select for more palatable forage.

Scanning electron microscopy images also revealed that the harsh variety of *F. arundinacea* had more numerous and larger leaf spines than those on the leaf surface of the soft variety, and this was particularly noticeable at the leaf margin. These spines may act as a deterrent to herbivores, especially cattle as they use their tongues to wrap around the blades of grass prior to chewing; if the grass feels spiny then it is likely to seem unpalatable to the cattle. An impact of Si levels on the bite rate of ruminants has been demonstrated experimentally for sheep (Massey et al., 2009), suggesting Si does impair food processing time. The soft variety had far fewer and smaller spines, suggesting these plants are depositing Si in a different way to the harsh variety. Even when not deposited as abrasive spines, Si can still make plants hard to digest, if for example these deposits prevent herbivores crushing cells to extract nutrients, as hypothesized by Hunt et al. (2008).

Contrary to our predictions, neither variety responded to the Si addition treatment with an increase in leaf Si content. This may be related to the young age of the plants and relatively short duration of the Si addition treatment. Silicon accumulation is influenced by transpiration rates, where older leaves are found to have significantly more Si than younger leaves, primarily due to Si translocation via the transpiration stream (Piperno, 2006). Furthermore, once deposited Si is not remobilized (Richmond and Sussman, 2003), meaning foliar Si levels increase with both plant and leaf age (Reynolds et al., 2012). Although the mechanisms underpinning Si uptake and distribution in plants are still not fully understood, it has been demonstrated that plant species differ in the Si uptake ability of their roots and in the density of their root transporters, as well as in their capacity to upload Si to the xylem (Ma and Yamaji, 2006). More recently, work on rice has suggested that shoots control the regulation of the Si transporters in the root and hence how much Si is taken up into the shoot (Yamaji and Ma, 2011). A study assessing the uptake abilities of over 500 plant species (Ma et al., 2001) classified them into high, intermediate and non- accumulators and it may be that *F. arundinacea* physiology is such that it is not a high accumulator of Si, even under conditions of high Si supply. It does however,

appear to be able to use the Si it does take up very efficiently in terms of spine production, at least in the case of the harsh variety. The mean Si values reported here for *F. arundinacea* are lower than those reported for this species in Hodson et al. (2005), which may reflect differences in age of the plants when sampled or the growing conditions of the plants. However the variation in foliar Si content shown in this species and indeed in other taxa within the genus (Hodson et al., 2005; Massey and Hartley, 2006; Massey et al., 2006, 2007a, 2009) suggests a high degree of phenotypic plasticity in the levels of Si seen within the leaves of *Festuca* species. This is perhaps unsurprising given the numerous factors, including plant genotype, biotic stresses such as herbivory and abiotic ones such as water availability, known to affect Si levels in plants (Soininen et al., 2013; Quigley and Anderson, 2014).

2.14.2 Interspecific Differences

Our hypothesis that abrasive species would have higher foliar Si concentration than less abrasive species was not well-supported: *D. cespitosa* has previously been reported to have high leaf Si and to be more abrasive than either of the *Festuca* species, but in this study had lower leaf Si concentration than *F. ovina* and *F. rubra*. Differences in experimental conditions, and hence in plant growth rate, and in plant age, size, and genotype (Soininen et al., 2013) may explain changes in the relative Si concentrations between species, but it is clear that our second hypothesis, namely that more abrasive species had larger, sharper and/or a greater number of spines and phytoliths is supported (Figure 2.5). SEM images revealed that *D. cespitosa* leaves are covered in Si-rich leaf spines which were absent from the leaf surfaces of *F. rubra* (and from *F. ovina* in the absence of additional Si). This strongly suggests that the leaf spines were significantly influencing the abrasiveness of *D. cespitosa* and that phytolith morphology may be more important than leaf Si concentration in determining the abrasiveness of leaves and thus the effectiveness of anti-herbivore defense.

There was a change in morphology and an increase in the number of Si-rich bodies deposited on the leaf surface of *D. cespitosa* and *F. ovina* when plants were provided additional Si; in the case of *D. cespitosa*, phytoliths which had not been present in control leaves were deposited (silica short cells), whereas for *F. ovina*, new, Si-enriched spines were produced, again when spines were not apparent on control leaves. This suggests that these plant species have the ability to deposit new types of Si-based structures to potentially increase their anti-herbivore defenses, whether via abrasion, digestibility effects or both, when Si supply is increased. These changes were most obvious when leaves were also damaged, although interestingly damage in the absence of additional Si did not produce them. In addition to changes in the nature of the spines, the EDX demonstrates that *D. cespitosa* deposited Si only at the tips of spines under control conditions, but under the Si addition treatment, the spines contain Si throughout and the leaf surface is also heavily silicified. A similar pattern was observed in *F. ovina* (Figure 2.5).

Our results support our predictions about the influence of Si supply on the level of Si-based defenses (also see Garbuzov et al., 2011), but damage had less effect on Si-based defenses than we predicted. Although damaged plants were found to have an increase in leaf Si concentration, this was far smaller than the effect of Si supply and there was little effect of damage on spine formation. This may reflect the fact that we used a clipping treatment; simulated damaged may not bring about the same response in spine/phytolith morphology and Si accumulation as herbivory, as reported in previous studies (Massey et al., 2007b). *F. rubra* demonstrated a greater Si uptake to damaged leaves on damaged plants than other species did, which suggests plant species show differences in the way they distribute their Si between different plant parts in response to damage (and potentially other stresses). The mechanism for this is currently unknown, but there have been reports of between species differences in the ability to load Si into the xylem (Ma and Yamaji, 2006).

2.15 Conclusion

There were marked differences in the way that even grass species from the same genus deployed the Si they take up in terms of its deposition in structures likely to affect their anti-herbivore defenses. Differences in the localization and the Si-based structures formed has been demonstrated before between plant families (Currie and Perry, 2007), but to our knowledge this is the first time such striking variation has been observed between grass species from the same genus. *F. rubra* had the highest foliar Si content and deposited more Si in damaged leaves than the other two species when plants were damaged under conditions of increased Si supply. However, it is the least abrasive species, presumably because its Si is deposited smoothly and evenly on the leaf surface and not in spines, and any phytoliths produced are few in number and, in contrast to spines, do not protrude substantially above the leaf surface. *D. cespitosa* has a very different strategy: a lower foliar Si content which was less affected by damage and Si addition, but what Si was present was deposited in numerous large spines, particularly at the tip, and under conditions of high Si supply, in a high density of additional structures which are absent under low Si supply. These structures may explain why this species has frequently been shown to be abrasive and unpalatable. Our results suggest that quantifying leaf Si concentration will not give a complete understanding of Si-based anti-herbivore defenses; rather examining how that foliar Si is deposited on the leaf surface will provide a better knowledge of how different plants use their Si and its likely impact on herbivores.

2.16 Supplementary analysis

2.17 Introduction

Festuca arundinacea Screb. (tall fescue) is a species of grass grown in Europe for animal forage and turf. A number of breeding varieties that are genotypically different have been developed by the commercial seed company, DLF Seeds Ltd. These varieties vary in a number of phenotypic traits including vulnerability to lodging, seed production, colour and leaf texture. Tall fescue is known for its rough, stiff, coarse leaves (Gibson and Newman, 2001) and grazing animals find it unpalatable due to this coarseness (Deakins, 1979; Gibson and Newman, 2001). The traits involved in causing the recorded variation in leaf texture are currently unknown.

Silicon (Si) is deposited in the leaves of plants in varying concentrations (Hodson et al., 2005). Phytoliths are solid silica bodies (Strömberg et al., 2016) found on the surface of the leaves and may be abrasive (Massey and Hartley, 2009; Calandra et al., 2016). Trichomes (hairs that protrude from the epidermis) are also found on the leaf surface and may be silicified (Samuels et al., 1993). Both phytoliths and trichomes therefore contribute to leaf texture and the quantity, density and morphology of both are known to vary among taxa – both within and between species (Piperno, 2006; Strömberg et al., 2016). There have been few studies into the Si composition of tall fescue (but see Hodson et al., 2005) and even fewer on the intraspecific variation.

Previous studies on interspecific variation in the *Festuca* genus (Massey et al., 2007a) found that *F. ovina* and *F. rubra* accumulated similar levels of Si within the leaves, but *F. ovina* was more abrasive compared to *F. rubra*. This difference in abrasiveness led to the theory that Si-based defenses were influenced by both the amount of Si and more crucially, how the Si is used within the silicified cells, rather than the quantity of Si alone. This appears to be the case between species of *Festuca*, and it was hypothesised this may also be true intraspecifically. Tall fescue was chosen as a model system to test this hypothesis, as distinctive genotypes are available which vary in leaf texture but information on the uptake ability is lacking.

This study was designed to test the hypothesis that Si was, at least in part, contributing to the leaf texture associated with varieties described as harsh and soft. To accompany the leaf Si analysis, the study also investigated whether the concentration of Si in the leaves alone was responsible for the coarse leaf texture, or if structures on and within the leaf epidermis involving Si deposition were also contributing to differences in leaf texture.

In addition to the hypotheses tested in Hartley et al. (2015) which focused on types of Si-based defenses, previous studies have found that carbon (C) negatively correlates with Si

concentrations (Cooke and Leishman, 2012) suggesting that there is a trade-off between Si and C-based defenses. In this study, the following hypothesis was also tested: plants that take up more Si will have less C, as C will be substituted for Si-based defenses (Cooke and Leishman, 2012).

2.18 Materials and Methods

The C and nitrogen (N) concentrations of leaf material (as% dry weight) were analysed by flash combustion and chromatographic separation of ~ 1.5 mg milled leaf material using an elemental analyser (Elemental combustion system 4010 CHNS-O Analyser, Costech Analytical Technologies, Inc., Milan, Italy), calibrated against the standard BBOT($C_{26}H_{26}N_2O_2S$) (Wade, 2015). Percentage C and N were converted to mg g^{-1}. Differences between varieties and plants without and with additional Si were tested in the statistical software R (version 3.0.2) using analysis of variance (ANOVA).

2.19 Results

No differences between C: N ratio were reported between variety or Si addition ($F_{1, 18}$ = 2.74, *P*= 0.12; $F_{1, 18}$ =0.03, *P*= 0.86). However, there was a difference between the amount of C between the two varieties: the soft variety had significantly more C in the leaves compared to the harsh variety ($F_{1, 18}$ = 4.89, *P*= 0.04). Leaf C concentration did not vary between plants without and with additional Si ($F_{1, 18}$ = 0.69, *P*= 0.42).

2.20 Discussion

In addition to the result presented in Hartley et al., 2015, it was found that C was significantly higher in the soft variety. This variety also had significantly lower Si concentrations compared to the H variety. Previous work found Si was negatively correlated with both C and phenolics (of which C is the main constituent) (Cooke and Leishman, 2012). Frew et al. (2016) also found Si was negatively correlated with phenolic concentrations in the root. These results provide evidence for an emerging area where little studies exist (Cooke et al., 2016) and suggest that trade-offs are involved in the uptake of Si by plants (Cooke and Leishman, 2012; Frew et al., 2016). Further work is necessary to understand the nature of these trade-offs, such as how cellulose and lignin are influenced by Si uptake and deposition, but see Chapter 3.

3 Chapter 3: Structural Si-based defenses in tall fescue

3.1 Introduction

Silicon (Si) is a highly abundant element found in the Earth's crust. Grasses accumulate between 1-10% Si (dry weight) in their tissues (Ma et al., 2006) and utilise Si as an anti-herbivore defense (Massey et al., 2006; Massey and Hartley, 2009; Hartley and DeGabriel, 2016). Silicon is incorporated within the cell wall, inter-and intracellularly, deposited as phytoliths (solid, amorphous Si bodies), and within trichomes (Piperno, 1988) leading to an unpalatable leaf texture for herbivores (Hanley et al., 2007; Laca et al., 2001). Allocation of resources to plant defenses, including those based on Si, varies both between and within species depending on a range of factors including the environmental resources available, the phylogeny and life history strategy of the species concerned, and the lifetime risk of herbivore attack (Coley, 1988; Jones and Hartley, 1999). These factors determine the most cost-effective defense strategy for a species or genotype in terms of minimising trade-offs between defense and growth, and between types of defense. In most grasses, the principal defenses are Si and carbon (C)-based defenses such as phenolics (Vicari and Bazely, 1993); allocation to these defenses has been shown to differ between species (Massey et al., 2007a; Cooke and Leishman, 2011b). Although allocation to C-based defenses such as phenolics is generally reduced in species with high growth rates (Imaji and Seiwa, 2010), evidence for an impact of allocation to Si defenses on plant growth remains scant: Si defenses appear to bring benefits in terms of resistance to herbivory and other stresses (Massey and Hartley, 2006; Guntzer et al., 2012; Cooke and Leishman, 2016, but see Simpson et al., 2017) without significant negative consequences for growth. There is however, increasing evidence for trade-offs between C-based defenses and Si (Cooke and Leishman, 2011b; 2012; Frew et al., 2016) suggesting there is a careful balance at play between the investment of these defenses within the plant. Thus far, little attention has been paid to the potential trade-offs between different types of Si-based defenses, how those trade-offs differ between plant genotypes, and how they are affected by herbivory.

Phytoliths are morphologically diverse both intra and interspecifically (Piperno, 1988). Recently, Si deposition in sedges has been shown to physically abrade the guts of the voles (Wieczorek et al., 2015), and another study showed that a greater phytolith density caused more microwear to the teeth of voles (Calandra et al., 2016), revealing the importance of phytoliths in Si-based inducible defenses in plants. Trichomes and phytoliths form the main physical structures for leaf Si deposition, and increase in response to herbivory (Traw and Dawson 2002; McLarnon et al., 2017), yet it is not known how an increase in the numbers of these structures in

response to herbivory alters the amount of Si in the leaf overall and how these changes influences other leaf traits, such as digestibility.

Other leaf traits associated with the cell wall also act as anti-herbivore defenses. Plant cell walls are mainly composed of cellulose, hemicelluloses and lignin (Zhang et al., 2015). In the cell wall, lignin binds covalently to hemicelluloses and covers cellulose microfibrils which increase the tensile strength of the cell walls (Murozuka et al., 2014). Cell wall polymers such as lignin and hemicelluloses are ester or ether-linked to phenolic acids (such as ferulic acid (FA) and *p*-coumaric acid (pCA)), and these bonds make the cell wall difficult to digest (Theander et al., 1981; Kondo et al., 1990; Grabber et al., 2004). Neutral detergent fibre (NDF) is the indigestible biomass remaining after treatment with cellulase enzymes and is mainly composed of plant cell walls – i.e. lignin, hemicelluloses and cellulose and as such is a measure of indigestibility. Neutral cellulase fibre (NCF) is the biomass remaining after treatment of remaining NDF biomass with cellulases, in order to digest remaining cellulose fractions, providing a measure of indigestibility of components other than cellulose. Silicon is deposited in the secondary cell wall, where its function is poorly understood, but thought to be structural i.e. providing rigidity and strength (He et al., 2013).

The interaction of Si with other structural traits in leaves has been poorly studied. Evidence suggests that FA cross-links may have a role in alleviating biotic stresses in plants (Barros-Rios et al., 2011; Bergvinson et al.1997; Buanafina and Fescemyer, 2012) and the reduction of ferulate accumulation led to increased susceptibility of herbivory by larval fall armyworm (Buanafina and Fescemyer, 2012). Recently, it has been reported that Si may bind to arabinoxylan-FA complexes, reducing the ability of FA to attach to lignin precursors such as monolignols (Soukup et al., 2017). Conflicting evidence regarding the effects of Si on lignin and cellulose concentrations have been reported in Si accumulating species (Schoelynck et al., 2010; Cooke and Leishman, 2012; Schoelynck and Struyf, 2016). Further, potential trade-offs between lignin and Si have been reported (Suzuki et al., 2012; Yamamoto et al., 2012; Soukup et al., 2017) but little evidence exists to explain the mechanisms underpinning these potential trade-offs. Silicon clearly interacts with these leaf traits, however, trade-offs involved in the allocation of leaf structural traits and Si have not yet been investigated.

Festuca arundinacea Schreb. (tall fescue) is a common forage and turf grass (Gibson and Newman, 2001), widely grown in Europe (Hand et al., 2012b). It accumulates Si (Hodson et al., 2005; Hartley et al., 2015), although less is known in relation to how this species distributes Si in relation to structural defenses. Previous work (Hartley et al., 2015) has shown intraspecific differences in relation to Si uptake and deposition. However, quantification of phytolith and trichome density over a range of varieties within this species and how these physical defenses relate to leaf Si concentration in terms of potential trade-offs has not yet been tested. How the

species responds to leaf damage by altering leaf traits such as FA, pCA, lignin, NDF and NCF in relation to Si has not yet been tested either. The findings from this study will reveal insights into how these traits contribute to digestibility. This study tested the following hypotheses:

1. Leaf Si concentration will increase in response to increases Si supply and damage.
2. As active Si uptake requires energy, there will be a trade-off between growth (in terms of total biomass) and Si concentration, reflected in a negative correlation between total biomass and leaf Si concentration.
3. Varieties described as harsh (assigned by plant breeders) will deposit more Si into the phytoliths and trichomes in response to increased Si supply and damage than the soft varieties.
4. Plants that respond to damage and additional Si supply by increasing leaf Si concentrations will decrease concentrations of other traits involved in digestibility such as FA, pCA, lignin, NCF and NDF.

3.2 Methods

3.2.1 Plant growth

Six varieties of *F. arundinacea* (very very soft (VVS), very soft (VS), soft (S), semi-soft (SS), semi-harsh (SH) and harsh (H)) were grown individually from seed in a loam based compost (John Innes No.2) in 13cm pots. Once established, plants were randomly assigned to 4 treatments: no Si or damage, Si addition, damage, Si addition and damage. Treatments were imposed 3 weeks after sowing, with plants harvested 9 weeks later. There were 8 replicate plants of each treatment combination for each variety except H with Si addition and damage which had 7 replicates, due to one replicate flowering and so was excluded from the analyses.

For all varieties, Si addition was achieved by watering plants with 150 mg L^{-1} solution of dissolved sodium metasilicate ($Na_2SiO_3.9H_2O$) in deionised water. Plants were watered 100ml twice a week with or without Si dissolved in deionised water and tap water added as required. Plants were mechanically damaged using a metal file twice a week over 9 weeks. Approximately half of the leaves on the plant were damaged and approximately 25% of the leaf was filed for 5 seconds during each treatment; leaves were damaged approximately half way down the leaf where possible. The remaining leaves were left undamaged.

3.2.2 Silicon analysis by portable X-Ray Fluorescence spectrometry (P-XRF)

Silicon was analysed by portable XRF, calibrated using Si-spiked synthetic methyl cellulose and validated using Certified Reference Materials of NCS DC73349 'Bush branches and leaves' obtained from China National Analysis Center for Iron and Steel (Reidinger et al.,2012).

Leaf material was ball milled (Retsch MM 400, Haan, Germany) for 2 minutes at frequency of 30 Hz (60 min^{-1}) with steel grinding balls. Leaf material was pressed at 10 tons into pellets with a manual hydraulic press using a 13 mm die (Specac, Orpington, UK). Silicon analysis (% Si DW) was performed using a commercial P-XRF instrument (Niton XL3t900 GOLDD analyser: Thermo Scientific Winchester, UK) held in a test stand (SmartStand, Thermo Scientific, Winchester, UK).

3.2.3 Epidermal peels

Epidermal peels were taken for 3 replicates per treatment per variety (n = 72). Clear nail polish was applied to the upper leaf surface; upon drying this was peeled off and fixed onto a microscope slide and analysed via Nikon Eclipse Ni-U light microscope (Nikon Instruments, Kingston Upon Thames, Surrey) for trichome and phytolith numbers.

3.2.4 Ferulic acid and pCA analysis

Ferulic acid and pCA concentrations of 3 leaf samples per treatment were determined by high performance liquid chromatography (HPLC). 10 mg of leaf biomass was flushed with Aragon gas and 1M NaOH. Samples were incubated at 25 °C for 16 hours before adding 100 µl of 99% TFA, 0.5ml 1-butanol. The samples were spun in a centrifuge at the highest speed for 5 minutes, the organic phase was recovered and the butanol extraction was repeated twice more, with the butanol evaporated each time. The remaining pellet was dissolved in 100% methanol and analysed using HPLC over a 20-70% methanol gradient.

3.2.5 Neutral detergent fibre and NCF

Neutral detergent fibre content of 4 leaf samples per treatment was determined using the filter bag technique (ANKOM Technology Method 9). Leaf samples were milled using a cutter mill

(MF10 basic, IKA WERKE) with a 1mm screen. Filter bags (F57, ANKOM Technology) were weighed and 300 mg of material was inserted. The filter bags were sealed using a heat sealer (1915 ANKOM Technology). Samples were placed into the fibre analyser vessel and submerged. Samples were saturated with neutral detergent solution – to this 20 g Na_2SO_3 and 4ml of pure alpha amylase solution and 8ml alpha amylase dissolved in 300 ml deionised water were also added. When cycle finished, bags were dried using acetone, air-dried and completely dried in an oven at 102 °C for 2 hours. Bags were placed in a desiccant pouch and bags were weighed again. The NDF was calculated using the following formula $\frac{\left(W3-(W1xC1)\right)}{W2}x100$; where W1=bag tare weight, W2=sample weight, W3=dried weight of bags, C1=blank bag correction.

Neutral cellulose fibre content of 4 leaf samples per treatment was determined using the samples from the NDF method. Samples were placed into preheated (to 39.5 °C) digestion jars (for ANKOM Daisy II Incubator) containing 2 litres of acetate buffer with 0.5 g cellulose and 0.1 g chloramphenicol. After 40 hours, samples were removed, washed three times with hot water (90 °C), rinsed with acetone and air dried. Once air dried, samples were placed in an oven at 105 °C for 2 hours, then placed in a desiccant pouch and weighed. The NCF content was then calculated by $\frac{\%NDF-\%NCF}{\%NDF}x100$.

3.2.6 Lignin

Lignin concentrations of 3 leaf samples per treatment were determined using the acetyl bromide method. 250 µl of 25% v/v acetyl bromide/ 75% glacial acetic acid) was added to 5 mg of sample and incubated at 50 °C for 3 hours. Samples were added to a 5 ml volumetric flasks, 1 ml 2 M NaOH was added to the tubes to get additional acetyl bromide and transferred to the flasks along with 175 µl of 0.5 M hydroxylamine HCL, flasks were filled to 5 ml with glacial acetic acid and mixed. 100 µl of the sample was added to a cuvette with 900 µl of glacial acetic acid. The absorbencies of each sample were measured at 280 nm.

3.2.7 Statistical analyses

Biomass measurements were based on dry weight (DW). All statistical analyses were performed using R (version 3.1.0). Analysis of variance (ANOVA) was used to test the main and interactive effects of variety, Si addition and damage on leaf Si concentration, trichome

density, phytolith density, FA concentration, pCA concentration, lignin concentration, NDF (% dry matter) and NCF (% dry matter). Linear models were used to check for normality and homogeneity of variance following Crawley (2007). Paired t-tests were used to compare the leaf Si concentration in varieties between plants without and with Si addition, and damaged and undamaged plants. Silicon, lignin, NDF and NCF (%) values were transformed using the arcsine square root transformation to meet the assumptions of the tests. Significance was set at $P<0.05$ for all analyses. *Post hoc* Tukey tests were carried out and significance was set at $P<0.05$. Packages used for analyses were as follows: lsmeans package (Lenth, 2016), ggplot2 package Wickham, 2009), multcompView package (Graves et al., 2015).

3.3 Results

3.3.1 Defense and growth

Leaf Si concentrations were significantly positively affected by adding Si to the growth medium in the following varieties: VS + 14% Si ($F_{1, 30} = 12.18$, $P = 0.002$), SS + 10% Si ($F_{1, 30} = 4.89$, $P = 0.03$) and H + 18% Si ($F_{1, 29} = 8.43$, $P = 0.007$); the other three varieties did not respond to additional Si supply by significantly increasing leaf Si concentrations: VVS ($F_{1, 30} = 1.66$, $P = 0.21$), S ($F_{1, 30} = 0.54$, $P = 0.47$) and SH ($F_{1, 30} = 0.56$, $P = 0.46$) (Figure 3.1).

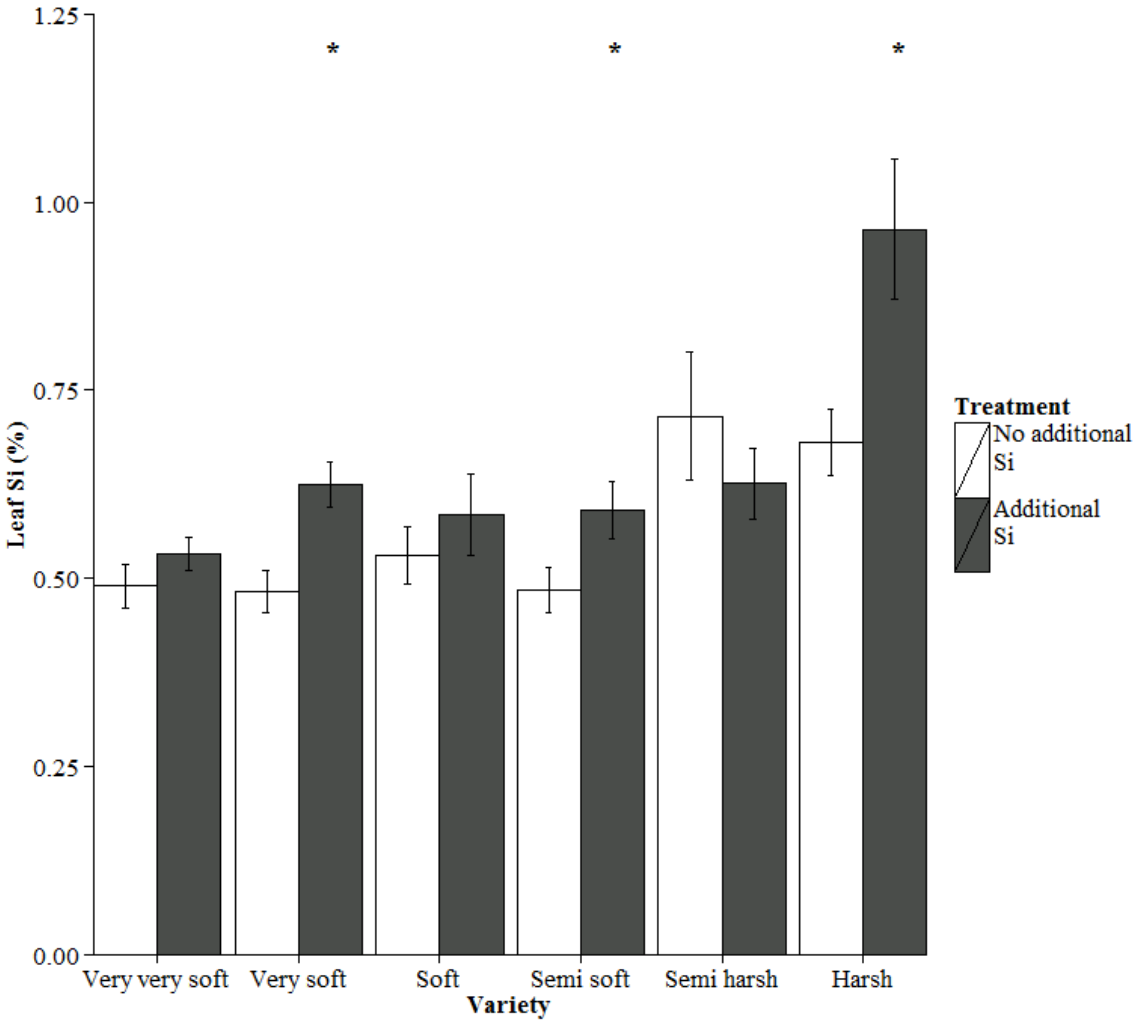

Figure 3.1: Leaf Si concentration of six varieties of tall fescue without and with additional Si; N = 16 per variety. Bars are mean values ± SE. Asterisks (*) denotes a significant increase in leaf Si concentration from no additional Si in response to additional Si within each variety.

Damaging plants influenced the leaf Si concentrations by causing a significant increase in the Si taken up and deposited in the leaves in the following varieties: VVS + 11% Si ($F_{1, 30}$ = 14, P <0.001), S + 20% Si ($F_{1, 30}$ = 15.28, P <0.001) , SS + 10% Si ($F_{1, 30}$ = 4.89, P = 0.03), SH + 31% Si ($F_{1, 30}$ = 27.63, P <0.001) and H + 18% Si ($F_{1, 29}$ = 8.57, P = 0.007); the other varieties did not respond to damage by significantly increasing leaf Si concentrations: VS ($F_{1, 30}$ = 1.75, P = 0.20) and SS ($F_{1, 30}$ = 2.48, P = 0.13) (Figure 3.2).

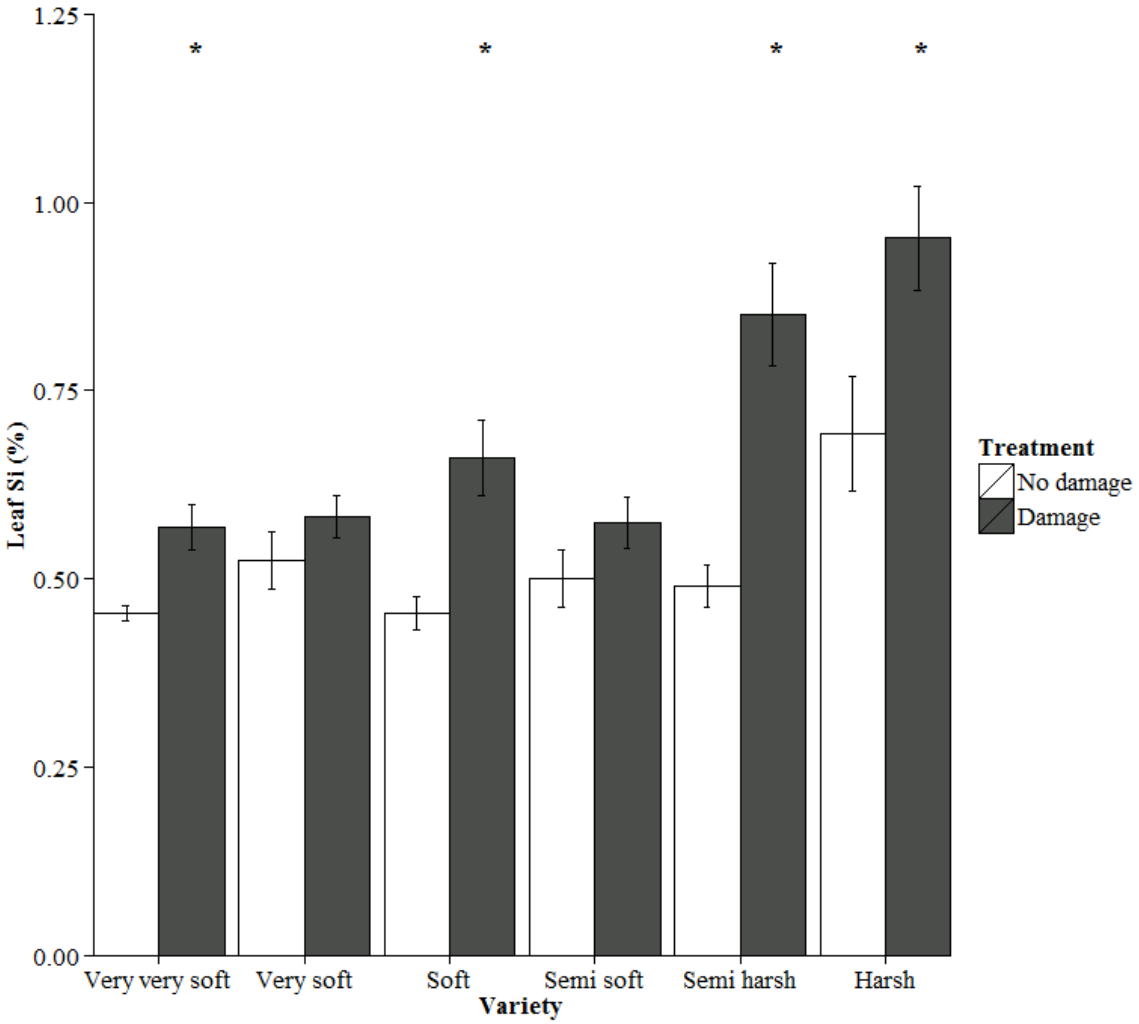

Figure 3.2: Leaf Si concentration of six varieties of tall fescue without and with plant damage; N = 16 per variety. Bars are mean values ± SE. Asterisks (*) denotes a significant increase in leaf Si concentration from undamaged plants to damaged plants within each variety.

To elucidate any biomass dilution effects of Si accumulated in the leaves in varieties with smaller growth phenotypes, Si uptake ability of the varieties was also tested. Overall, significant main effects were found in both variety ($F_{5, 167}$ =5.30, P<0.001) and damage ($F_{1, 167}$ =11.92, P<0.001), and an interaction between variety*damage ($F_{5, 167}$ =2.44, P=0.04) (Figure 3.3) showed the varieties responded to damage differently. These differences within variety, between undamaged and damaged plants (in terms of significant changes in Si uptake ability) increased in the following varieties: VVS + 30% Si ($F_{1, 30}$ =4.99, P=0.03), SH + 71% Si ($F_{1, 30}$ =14.17, P<0.001) and H + 58% Si ($F_{1, 29}$ =5.86, P=0.02). The Si uptake ability of the other three varieties remained unchanged when damaged (Figure 3.3).

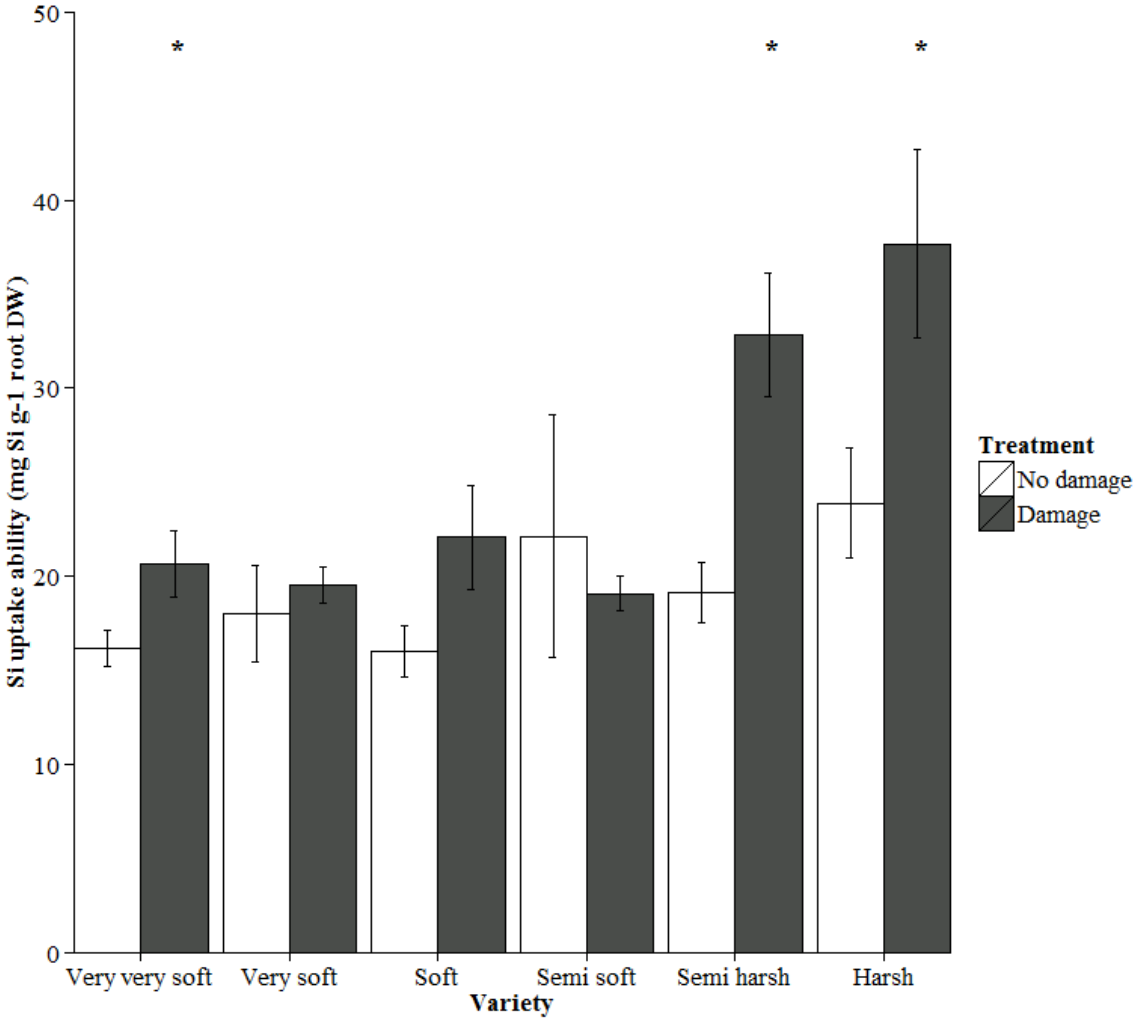

Figure 3.3: Si uptake ability (per milligram of Si per gram of root) in six varieties of tall fescue without and with plant damage; N = 16 per variety. Bars are mean values ± SE. Asterisks (*) denotes a significant increase in Si uptake ability from undamaged plants to damaged plants within each variety.

Total biomass was reduced in plants where leaf Si concentrations were high, resulting in a significant negative correlation between leaf Si concentration and total plant biomass (n = 191, r = -0.52, P<0.001, Figure 3.4). The H variety had the smallest total plant biomass ($F_{5,167}$= 4.96, P<0.001, *post hoc* Tukey P<0.05) and the lowest stem DW ($F_{5,167}$=9.71, P<0.001, *post hoc* Tukey P<0.05) compared to the four soft varieties (Table 3.1). Damaged plants had significantly lower stem DW compared to undamaged plants ($F_{1,167}$ = 6.19, P = 0.01). Plants receiving additional Si in the growth medium, that responded to additional Si supply (Figure 3.1) altered their biomass allocation by significantly increasing the root to shoot ratio when compared with those not receiving additional Si ($F_{1,167}$ = 5.78, P = 0.02).

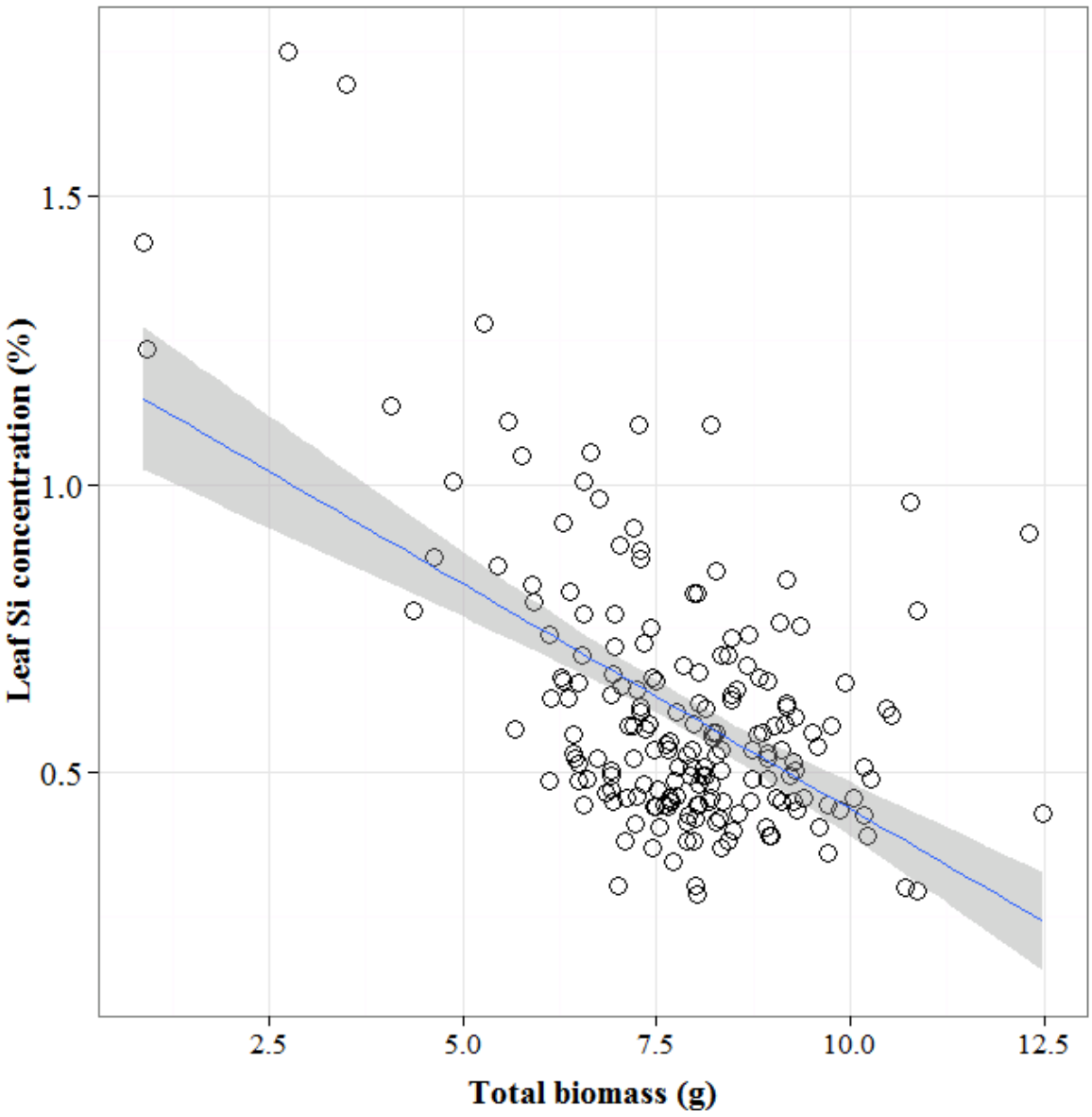

Figure 3.4: Correlation between leaf Si content (%) and total plant biomass (g) for all plants. Solid line represents linear regression through the points. Shaded area represents 95% confidence intervals.

Table 3.1: ANOVA statistics for leaf Si concentration and biomass of all varieties. Variety d.f.= 5, 167; Si addition d.f. = 1, 167; damage d.f. = 1, 167; variety* Si addition d.f. = 5, 167; variety* damage d.f. = 5, 167. Statistically significant results are highlighted in bold.

Measurement	*F* value					*P* Value				
	Variety	Si addition	Damage	Variety* Si	Variety* Damage	Variety	Si addition	Damage	Variety* Si	Variety* Damage
Leaf Si concentration (%)	**16.28**	**16.54**	**67.18**	**3.89**	**4.05**	**<0.001**	**<0.001**	**<0.001**	**<0.005**	**<0.005**
Si uptake ability (mg Si g-1 root DW)	**5.30**	0.06	**11.90**	0.79	**2.44**	**<0.01**	0.81	**<0.001**	0.56	**0.04**
Total biomass DW (g)	**4.96**	0.0005	1.59	1.52	0.87	**<0.001**	0.98	0.21	0.19	0.5
Stem DW (g)	**9.71**	0.13	**6.19**	1.36	0.54	**<0.001**	0.72	**0.01**	0.24	0.74
Root: Shoot	1.94	**5.78**	0.82	1.92	0.33	0.09	**0.02**	0.37	0.09	0.89

3.3.2 Structural defense

The varieties that responded to Si addition in the growth medium (VS, SS and H) and to damage (VVS, S, SH, H) by increasing leaf Si concentrations, were further analysed to test hypothesis three: varieties described as harsh (assigned by plant breeders) will deposit more Si into the phytoliths and trichomes in response to increased Si supply and damage than the soft varieties.

3.3.2.1 Without and with additional Si supply

Neither trichome density nor phytolith density were related to leaf Si concentration: no correlations were reported between trichome density and leaf Si concentration (n = 33, r = 0.30, P = 0.09) and phytolith density and leaf Si concentration (n = 33, r = -0.20, P = 0.28). Adding Si to the growth medium led to a higher trichome density compared to those without additional Si supply ($F_{2, 26}$ = 9.07, P = 0.006; Table 3.2). The SS variety had a higher phytolith density compared to the H variety ($F_{2, 26}$= 3.80, P = 0.04; Figure 3.5). No interactions between Si supply and variety were found.

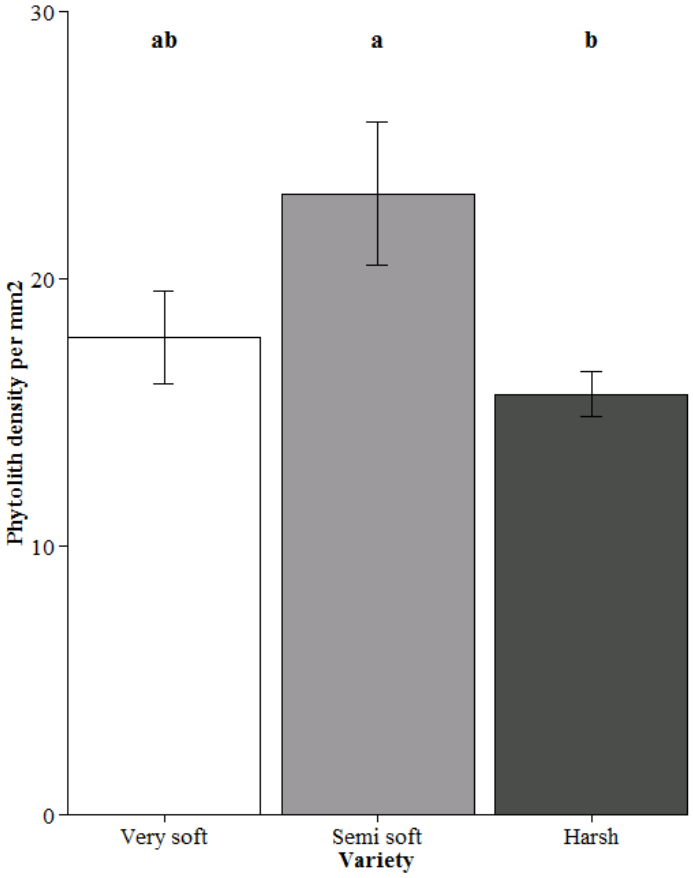

Figure 3.5: Phytolith density (per mm^2) in three varieties of tall fescue that responded to Si addition; N = VS:11 ; SS: 11; H: 10. Bars are mean values ± SE. Letters represent significant increase in phytolith density within each variety.

Table 3.2: Leaf trichome and phytolith densities in varieties that increased leaf Si concentration with and without additional Si in the growth medium. Values represent mean ± SE.

Variety	Phytolith density (per mm^2)		Trichome density (per mm^2)	
	Si-	**Si+**	**Si-**	**Si+**
VS	15.33 ± 1.89	20.80 ± 2.58	2.89 ± 1.47	11.39 ± 3.20
SS	24.20 ± 4.50	22.33 ± 3.57	2.77 ± 0.76	4.20 ± 1.59
H	15.17 ± 1.14	16.50 ± 1.19	5.86 ± 1.59	12.25 ± 3.62

3.3.2.2 Undamaged and damaged plants

There was a positive correlation between trichome density and leaf Si concentration (n = 33, r = 0.31, P = 0.04). There was a negative correlation between phytolith density and leaf Si concentration, although this was marginally non-significant (n = 33, r = -0.29, P = 0.06). The H variety had a higher trichome density than the VVS and S variety ($F_{3, 36}$ = 4.82, P = 0.006; Table 3.3). No differences were found between undamaged and damaged plants, nor were any interactions found between variety and damage. The S variety had a significantly higher phytolith density compared to the VVS variety ($F_{3, 36}$ = 3.35, P = 0.03; Table 3.3).

Table 3.3: Leaf trichome and phytolith densities in varieties that increased leaf Si concentration when plants were undamaged (D-) or damaged (D+). Values represent mean ± SE.

Variety	Phytolith density (per mm^2)		Trichome density (per mm^2)	
	D-	**D+**	**D-**	**D+**
VVS	15.00 ± 1.65	14.60 ± 2.58	1.93 ± 0.80	1.29 ± 0.70
S	24.00 ± 3.10	21.20 ± 2.08	2.07± 1.31	4.57 ± 2.33
SH	20.17 ± 3.74	17.17 ± 3.73	4.92 ± 0.96	4.02 ± 1.88
H	15.60 ± 1.03	15.80 ± 1.39	7.72 ± 2.68	9.12 ± 3.03

3.3.3 Other Digestibility Traits

The varieties that responded to the Si addition (VS, SH and H) and damage (VVS, S, SH, H) treatments, by changing their leaf Si concentrations, were further analysed to test hypothesis four: plants that respond to damage and additional Si supply by increasing leaf Si concentrations, will decrease concentrations of other traits involved in digestibility such as FA, pCA, lignin, NCF and NDF.

3.3.3.1 Without and with additional Si supply

Ferulic acid concentration was positively associated with high leaf Si concentrations (P = 0.02, r= 0.40, n = 72). Lignin showed the opposite relationship with high leaf Si concentration, with a negative correlation between leaf Si concentration and lignin concentration (P = 0.03, r = -0.36, n = 72). No other correlations were reported.

The SS variety had significantly lower leaf concentrations of FA compared with the VS and H varieties ($F_{2, 30}$ = 11.22, P < 0.001; Figure 3.6) and an interaction between variety *Si addition which showed that the SS variety reduced FA concentration in response to additional Si, whereas the other two varieties showed a trend for increased FA concentration in response to additional Si, though these responses were not significant (Figure 3.6). Plants given additional Si had lower concentrations of pCA in the leaves compared to those treated without additional Si ($F_{1, 30}$ = 4.84, P = 0.05). The SS variety had higher concentrations of NDF compared to the VS variety, with the H variety having similar NDF concentrations to both VS and SS ($F_{2, 42}$ = 4.84, P = 0.05). No other significant relationships were observed (Table 3.4).

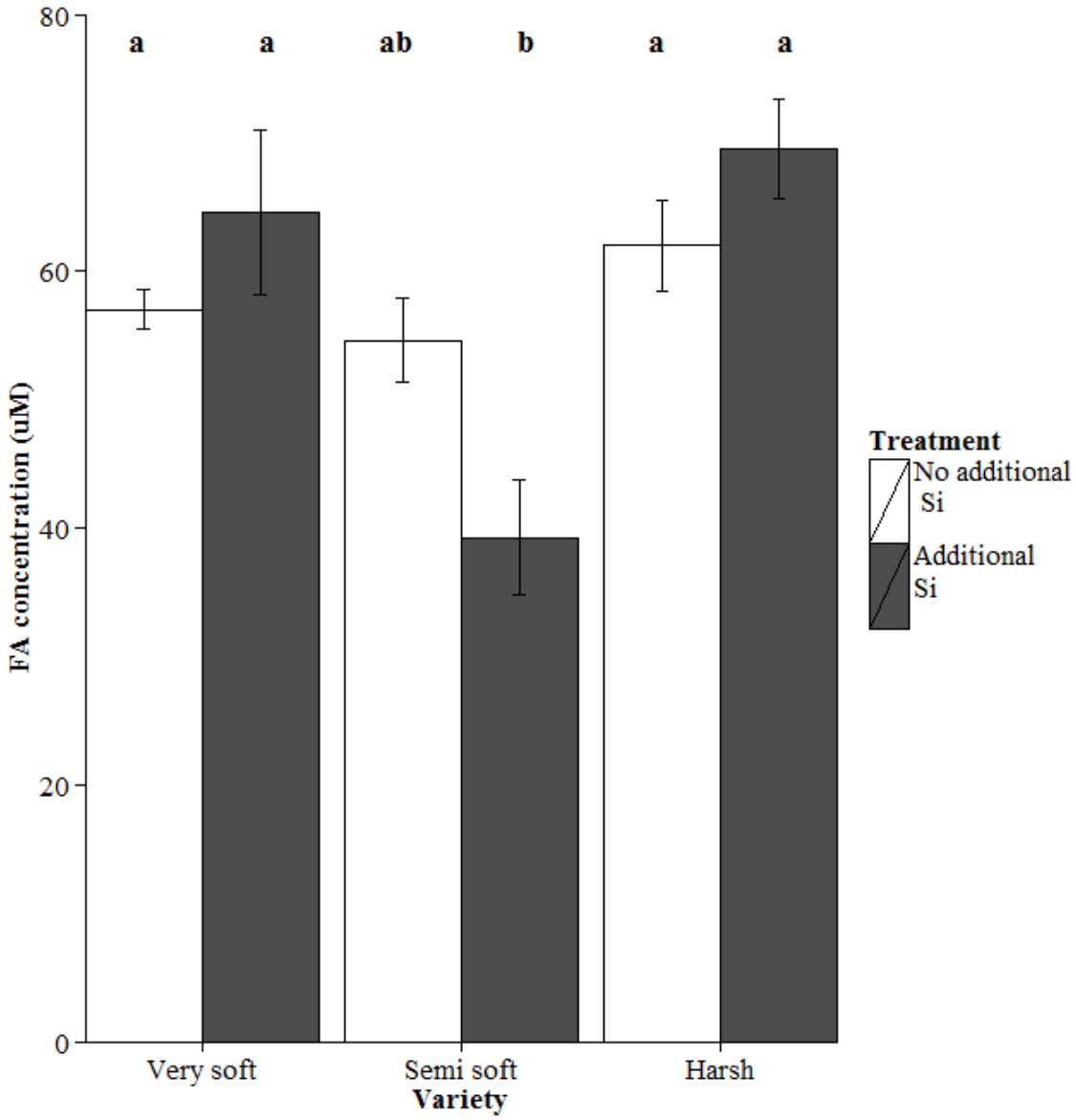

Figure 3.6: Ferulic acid concentration (µm) in three varieties of tall fescue; N = VS: 12 ; SS: 12; H: 12. Bars are mean values ± SE. Letters represent statistically significant bars (*post hoc* Tukey $P < 0.05$).

Table 3.4: ANOVA statistics for VS, SS and H varieties of plants without and with additional Si. Ferulic acid, pCA and lignin: variety d.f. = 2, 30; Si addition d.f. = 1, 30; variety* Si addition d.f. = 2, 30. Ferulic acid, NDF and NCF: variety d.f. =2, 42; Si addition d.f. = 1, 42; variety* Si addition d.f. = 2, 42. Statistically significant results are highlighted in bold.

Measurement	***F* value**			***P* value**		
	Variety	Si addition	Variety* Si	Variety	Si addition	Variety* Si
FA	**11.22**	0.00	**5.15**	**<0.001**	0.98	**0.01**
pCA	0.84	**4.12**	2.02	0.44	**0.05**	0.15
Lignin	1.27	0.86	1.76	0.29	0.36	0.19
NDF	**3.61**	0.09	1.92	**0.04**	0.77	0.16
NCF	0.63	0.09	0.05	0.54	0.76	0.95

3.3.3.2 Undamaged and damaged plants

Ferulic acid concentration was higher in plants with more Si deposited in the leaves (n = 72, r = 0.41, $P < 0.005$). No other correlations between Si concentration and pCA, lignin, NDF and NCF were found. The S variety had significantly less FA compared to the VVS, SH and H varieties ($F_{3, 40} = 20.10$, $P < 0.001$). There was also an interaction between variety*damage revealing that the damaged S plants had significantly less FA in the leaves compared to the H variety ($F_{3, 40} = 4.37$, $P < 0.01$; Figure 3.7) .

The SH variety has significantly more lignin compared to S, H and VVS and the S variety had significantly more lignin compared to the VVS variety ($F_{3, 40} = 24.79$, $P < 0.001$). Damaged plants were less digestible than undamaged plants, reflected in the higher NDF% in damaged plants compared with undamaged plants ($F_{1, 56} = 5.27$, $P = 0.03$). NCF% was significantly higher in the VVS variety compared with the H variety ($F_{3, 56} = 24.79$, $P < 0.001$;

Figure 3.8). No other significant relationships were observed (Table 3.5).

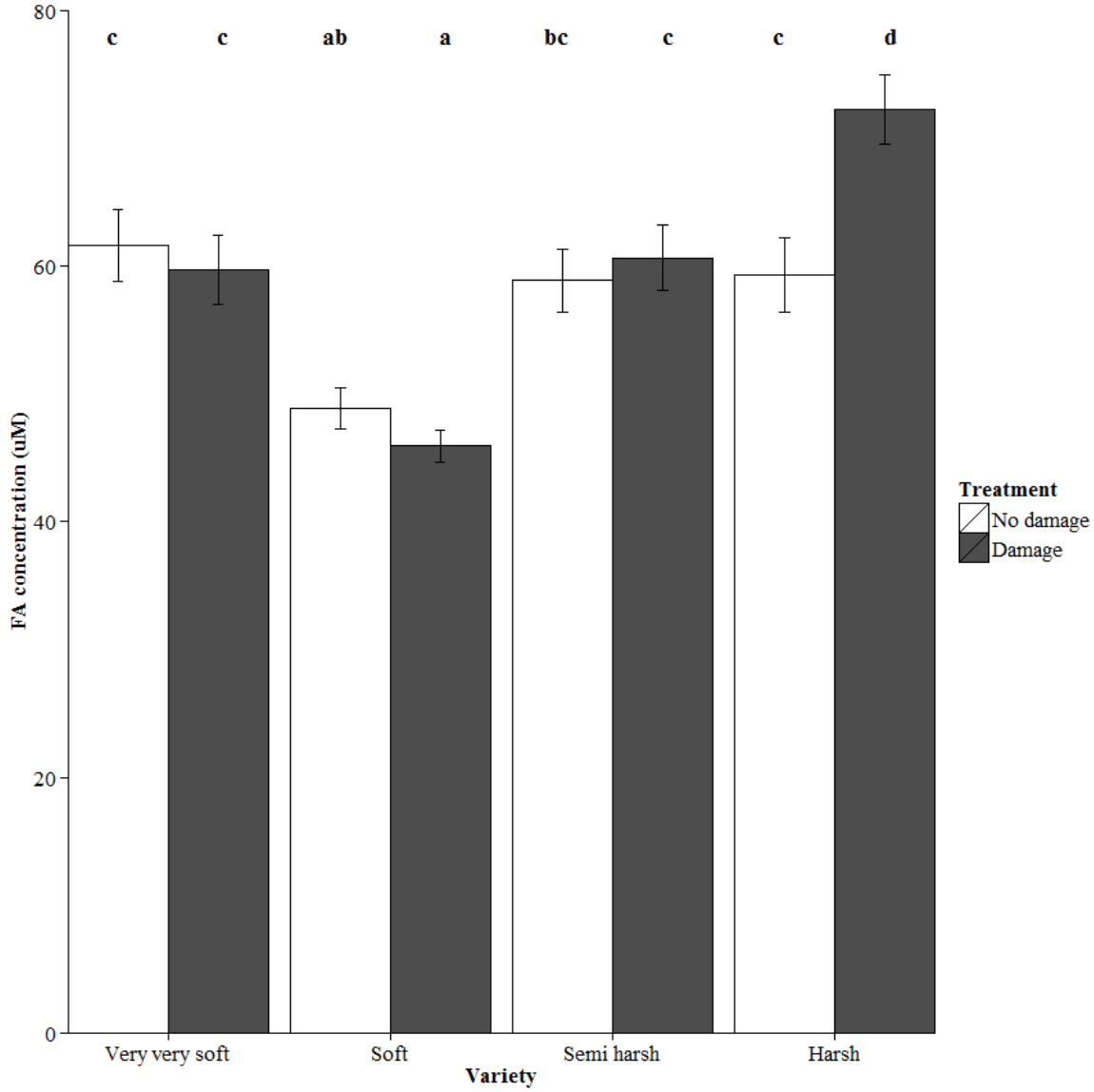

Figure 3.7: Ferulic acid concentration (µM) in four varieties of tall fescue; N = VVS: 12; S: 12; SH: 12; H: 12. Bars are mean values ±SE. Letters represent statistically significant bars (*post hoc* Tukey $P < 0.05$).

Table 3.5: ANOVA statistics for VS, SS and H varieties of plants without and with additional Si. Ferulic acid, pCA and lignin: variety d.f. = 3, 40; Si addition d.f. = 1, 40; variety* Si addition d.f. = 3, 40. Ferulic acid, NDF and NCF: variety d.f. = 3,56 ; Si addition d.f. = 1, 56; variety* Si addition d.f. = 3, 56. Statistically significant results are highlighted in bold.

Measurement	***F* value**			***P* value**		
	Variety	Damage	Variety* Damage	Variety	Damage	Variety* Damage
FA	**20.10**	2.01	**4.37**	**<0.001**	0.16	**0.01**
pCA	0.38	0.23	0.62	0.77	0.63	0.61
Lignin	**24.79**	0.13	0.43	**<0.001**	0.72	0.74
NDF	1.90	**5.27**	0.28	0.14	**0.03**	0.84
NCF	**3.47**	0.91	1.68	**0.02**	0.34	0.18

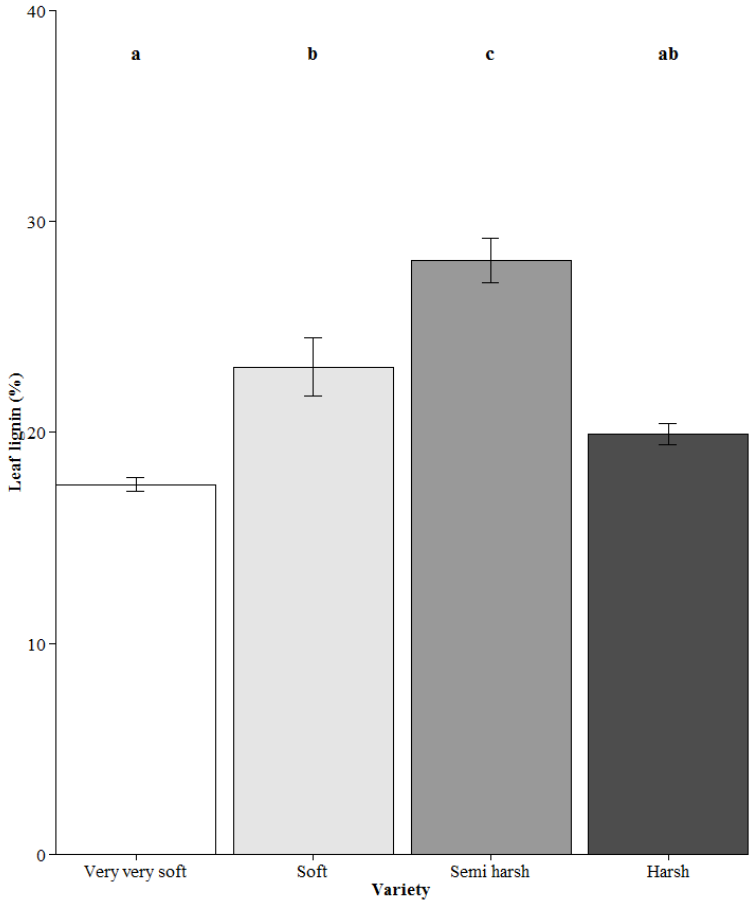

Figure 3.8: Leaf lignin % (dry matter) in four varieties of tall fescue; N = VVS: 12; S: 12; SH: 12; H: 12. Bars are mean values ± SE. Letters represent statistically significant bars (*post hoc* Tukey $P<0.05$).

3.4 Discussion

The varieties responded to both Si addition and damage differently: overall, the harsh variety was the most responsive to both treatments in terms of Si uptake and deposition, demonstrated by the increased leaf Si concentrations following treatments. The H variety also had the highest density of trichomes compared to the five other varieties and showed a trend for increasing trichome density by 18% in response to damage (though this was not statistically significant). Although the H variety had the smallest biomass DW (g), the Si uptake ability was not hindered: it was able to take up the most Si per gram of root DW compared to the four soft varieties when damaged. Compared to the varieties that responded to damage, the H variety contained less lignin (% DW) than the SH variety, yet had the highest FA concentration of the four varieties when damaged. The relationship between Si and other leaf traits appears to be complex: Si is tightly linked to FA in the H variety when the plants are under stress (i.e. damaged), evidenced by an increase in both FA and Si concentrations in the leaf when damaged, and a positive correlation between FA and leaf Si concentration. Ferulic acid is

converted to diferulic acid (DFA) in a peroxidase-catalysed coupling reaction (Hossain et al., 2007; Grabber et al., 1995; Ishii, 1991) and DFA is used to form cross links with cell wall polymers, such as lignin. Soukup et al. (2017) suggest a model to explain the trade-off between Si and lignin exists; Si forms links with arabinoxylan-FA complexes, reducing the links between lignin precursors and arabinoxylan-FA complexes. These findings suggest that, at least in the H variety, FA may be forming cross links with Si and impeding links with lignin and its precursors, which may explain the low concentrations of lignin in the H variety compared to the SH variety.

3.4.1 Leaf Si concentration increased in response to Si and damage

It was hypothesised that leaf Si concentration would increase in response to increased Si supply and damage. Varietal differences in leaf Si concentrations when additional Si was supplied and plants were damaged were found. Fewer varieties responded to additional Si supply than to damage treatments. Previous work (Hartley et al., 2015) showed that Si supply did not increase Si uptake in tall fescue, but differences in Si concentrations between varieties were noted: the H variety took up more Si than the soft variety. McLarnon et al. (2017) showed that the expression of the *Lsi2* transporter was upregulated in the H variety compared to the VVS variety. Therefore, it seems that genotypic differences in Si concentrations are, in part, driven by higher expression of Si transporters. Findings from Kumar et al. (2016) also support the idea that Si deposition in the leaf has an active element to it, but the exact mechanisms remain to be found.

3.4.2 Trade-offs occur between growth and Si uptake in varieties where Si uptake is higher

It was also hypothesised that due to the energy requirements of active Si uptake, there would be a trade-off between growth (in terms of biomass) and leaf Si concentration. Despite the H variety having the smallest biomass compared to the four soft varieties, when damaged, the H variety accumulated more Si (mg) per gram of root, compared to the four soft varieties. These results indicate a possible growth penalty, in terms of Si accumulation in this variety. Lsi2 is an efflux transporter, driven by a proton pump (Ma et al., 2007a) and therefore requires energy to actively pump Si into the stele, this may explain why the variety that accumulates the most Si out of all six varieties (H) has the smallest biomass, due to a trade-off in growth associated with the cost of active Si uptake. It has been previously suggested that anti-herbivore defenses may be traded-off for growth (Coley, Bryant and Chapin, 1985), since active Si uptake is metabolically expensive, it is possible that growth is reduced to compensate for the increase in

Si accumulation. McLarnon et al. (2017) showed there was an up regulation of the active transporter Lsi2, in the same variety of *F. arundinacea* in damaged conditions compared to the VVS variety, but no differences in the expression of the passive Si transporter, Lsi1. This suggests that the H variety invests more in active Si uptake compared to the other varieties and the smaller biomass may be the penalty incurred for increased Si uptake.

A difference in the root to shoot ratio when plants were given additional Si in the growth medium was found. This difference in biomass allocation suggests that root size (in terms of increased root DW) is important in Si acquisition and uptake. The change in biomass allocation suggests that with more root biomass available, a higher density of Si transporters may also available to take up more Si. Hattori et al. (2005) also found a significant increase in the root to shoot ratio in plants subjected to drought stress when Si was supplied. This suggests that Si accumulation is also influenced by environmental factors as well as genetic factors, and that root biomass has a significant role in Si accumulation, possibly through sensory mechanisms in the root via Si transporters, and this increase in root biomass may lead to an increase in Si transporter density. It may also be that with more Si available, more Si is taken up passively and deposited in the roots, causing the roots to become heavier. Silicon deposition in the root has been reported (Lux et al., 2003; Fleck et al., 2015) and increases in root DW in onions were reported when additional Si was supplied and more Si was taken up and deposited into the roots (Fleck et al., 2015).

It has been predicted that high Si accumulating species have reduced final plant mass compared to low Si accumulating species (Simpson et al., 2017). This predicted model, along with the reduced biomass in the H variety, demonstrates for the first time, a cost to the induction of Si defenses within a species.

3.4.3 Harsh variety invests more in trichomes, but not phytoliths

It was hypothesised that varieties described as harsh (assigned by plant breeders) would deposit more Si into leaf phytoliths and trichomes in response to increased Si supply and damage than the soft varieties. Silicon addition increased trichome density overall, compared with plants not given additional Si, but no varietal effects were found. These results are similar to those previously reported, where trichome density increased in response to Si supply in borage (Torabi et al., 2015), cucumber (Samuels et al., 1993) and soybean (deSouza et al., 2014).

Trichomes are known to be an effective anti-herbivore defense, particularly in protection against insect herbivores and production of trichomes is known to increase post-herbivory (Tian et al., 2012). Although no statistically significant differences were found between undamaged and

damaged plants, both the S and H varieties showed trends for increasing trichome production when plants were damaged and overall the H variety had the highest trichome density. These results suggest that varietal differences in trichome density is at least in part responsible for the leaf textures, especially as trichomes are known to be silicified (Hartley et al., 2015; Meunier et al., 2017). Meunier et al. (2017) reported fewer trichomes were produced in low Si conditions but more trichomes were produced when Si was readily available (at concentration 1.5mM hydroponically) and the results from this study show similar findings. They hypothesised that Si is stored in trichomes as a reservoir for additional Si (Meunier et al., 2017). This may well be the case in unstressed conditions, but this study found a negative correlation between phytolith density and leaf Si concentration in the varieties that responded to damage. If trichomes were acting as a storage facility for extra Si accumulated, it doesn't explain why trichome production increased and phytolith production decreased in the varieties that responded to damage in this study. Schaller et al. (2012) found a 25% decrease in phytolith deposition when plants received a higher Si dose compared to a lower dose. These results, combined with the increase in trichome density found at higher Si supply, suggests that there is a trade-off between the production of the structures involved in Si deposition. Trichomes appear to be produced when Si concentrations are higher and more Si is available (Samuels et al., 1993; deSouza et al., 2014; Meunier et al., 2017), suggesting these structures may be costly to make under lower Si conditions or that Si is being used elsewhere. These findings may explain why varieties with low Si concentrations in the leaves produce less trichomes and more phytoliths.

3.4.4 Ferulic acid and Si relationship suggests evidence for trade-off between Si and lignin

It was hypothesised that plants that responded to damage and additional Si supply by increasing leaf Si concentration, would decrease concentrations of other traits involved in digestibility such as FA, pCA, lignin, NCF and NDF. In the varieties that responded to additional Si supply, a positive correlation between FA and leaf Si concentration was found. In the cell wall FA is converted to DFA, which cross-links with cell wall polymers such as lignin (Grabber et al., 1995; Ishii, 1991; Hossain et al., 2002). Cell wall bound FA is known to decrease cell wall extensibility (Tan et al., 1991; Hossain et al., 2002) and previous studies have found a decrease in FA when Si is applied (Hossain et al., 2002; Goto et al., 2003; Fleck et al., 2015). Silicon is known for preventing lodging and is thought to make leaves more erect, thereby increasing photosynthetic capabilities (Ma et al., 2001). It is possible that some varieties of tall fescue utilise FA and Si in the cell wall to decrease extensibility to increase leaf erectness, and it has been suggested that Si deposition in plants grown in the presence of Si are more erect (Lwein and Reimann, 1969; Law and Exley, 2011; Strömberg et al., 2016) and that Si is useful for structural support. Further, it is thought that Si plays this role in horsetail (serving to rigidify the cell wall) by cross linking with cell wall polymers (Currie and Perry, 2009; He et al., 2013).

Many studies have looked into the effects of Si fertilisation/ supply (in an additive manner) on concentrations of phenolic acids in the leaf (Hossain et al., 2002; Goto et al., 2003; Fleck et al., 2015), but few have looked at the correlation between FA and Si - most studies report if FA concentrations increased or decreased in response to Si application. The positive correlation between FA and Si is contrary to the results in the literature, but when considered with the negative correlation between leaf Si and lignin concentration, it appears that Si may be substituted for lignin when Si supply is high, enabling a more favourable leaf C balance (Cooke and Leishman, 2012). Plants in the Poales use Si in the cell wall to maintain physical strength of the cell wall structures (Miwa et al., 2009; He et al., 2013) and it is thought that Si has a role in cross-linking cell wall components in monocotyledons (He et al., 2013). Leaf C was not measured here, but it may be possible that Si is being used structurally instead of lignin – and this may explain the positive relationship between FA and Si. This area remains poorly understood and further studies into lignin and Si are required to fully understand the relationship between these leaf traits.

In the varieties that responded to damage, damaged plants were less digestible, demonstrated by the higher NDF values. A study on rice lines that replaced FA with pCA show improved digestibility (Mitchell et al., 2007; Hatfield et al., 2017) as the pCA reduced the cross-links associated with FA in the cell wall. Silicon content in the cell wall is also known to reduce digestibility (Zhang et al., 2015) and a strong positive correlation between FA and Si concentration in the leaves was found here. These findings may explain why this study found reduced digestibility in the damaged plants compared with undamaged plants, as they had high levels of FA and Si.

3.5 Conclusions

Tall fescue varieties responded differently to Si addition and damage. Three out of the six varieties responded to Si addition by significantly increasing their leaf Si concentration and four of the six varieties responded to leaf damage by significantly increasing their leaf Si concentration. Despite having the lowest biomass, the H variety had the greatest Si uptake ability per gram of root. This study found that varieties with high Si concentrations had a growth penalty, in terms of overall total biomass. It was also found that Si addition caused a change in biomass allocation towards increased root biomass and this enabled more Si to be taken up into the leaves. Trichome density increased in response to damage and phytolith density decreased, suggesting a trade-off between these two structural defenses. Under low Si conditions, phytoliths are favoured and under high Si supply, more trichomes are produced, not previously found in any studies. Si and FA were positively correlated in the leaves, and may be contributing to low digestibility in damaged plants. The results are complex, but demonstrate

the plasticity involved in inducible Si defenses and suggest there is a cost involved in Si uptake in terms of biomass production, which has not yet been demonstrated – Simpson et al. (2017) predicted high Si accumulating species would have lower plant biomass and this is what was found intra-specifically. These results have important implications for the selection of traits involved in plant defense and show that Si supply and availability are also important drivers in the types of structural defense employed on the leaf surface in this species.

4 Chapter 4: Evidence for active uptake and deposition of Si-based defenses in tall fescue

4.1 Introduction

Silicon (Si) is considered a non-essential element, but it has many useful functions in plants (Guntzer et al., 2012). Plants take up Si in the form of monosilicic acid [$Si(OH)_4$] via the roots (Ma et al., 2006). It is transported through the xylem and deposited in the leaves to form phytoliths. Phytoliths are solid bodies of silica (SiO_2) found in epidermal layers, both within and between the plant cells (Piperno, 1988; Currie and Perry, 2007). Trichomes (small hairs found on the leaf surface) may also become enriched with Si and increase the abrasiveness of leaf surfaces. Plants within the grass family (Poaceae) accumulate Si in varying concentrations (up to 10% dry weight) where its primary function is to defend the leaf surface against a range of stresses including drought (Emam et al., 2014; Mitani-Ueno et al., 2016), pathogen attack (Fauteux et al., 2005; Liang et al., 2015) and herbivory (Massey et al., 2006, 2007b; Hartley et al., 2015). Many species of grass show diversity in their reported shoot Si concentrations (Ma et al., 2001; Hodson et al., 2005; Massey and Hartley, 2006; Hunt et al., 2008). Differences in the density and efficiency of Si transporters may underpin these differences, as reported in rice (Wu et al., 2006; Ma et al., 2007a), whilst environmental conditions such as water availability and herbivory can also drive changes in Si concentration (Quigley and Anderson, 2014; Wieczorek et al., 2015). The relative importance of genotypic, phenotypic and environmental factors for Si uptake remains unclear (Hartley et al., 2015; Hartley and DeGabriel, 2016).

Lsi1 is a root-specific Si transporter involved in the transport of Si from the soil solution [as $Si(OH)_4$] to within the root, first identified in rice (Ma et al., 2006), though orthologues of Lsi1 have now been identified in other crop species (e.g., *Zea mays* L, Mitani et al., 2009a,b; *Hordeum vulgare* L., Chiba et al., 2009; Yamaji et al., 2012 and *Glycine max* (L) Merr., Deshmukh et al., 2013). Lsi1 in rice is a passive aquaporin-like transmembrane protein (Yamaji and Ma, 2007) which transports Si into the root cells, whilst a Si efflux transporter, Lsi2, actively pumps (driven by a proton gradient) Si out of the root cells and into the stele (Ma et al., 2007a; Deshmukh and Bélanger, 2016). Aquaporins permit the passage of water through the cell membrane following the gradient in water potential, suggesting that Si can enter the plant cells without the need and use of Si specific transporters (Exley, 2015). Contrary to this, some studies have found that the Si transporters in rice (a hyper-Si accumulator, accumulating up to 10% Si in dry weight) and maize are down-regulated after constitutive Si supply (Yamaji and Ma, 2007; Mitani-Ueno et al., 2016), which would not be the case if the transport was purely via water

flow into the cells. Furthermore, some studies have reported tissue Si concentrations above that plausible for passive transport only (Faisal et al., 2012; Yamaji and Ma, 2014; Yamaji et al., 2015). Silicon has been identified in plant parts with low transpiration such as the husk, presumably actively redirected to these locations by Si-mediated transporters (Yamaji and Ma, 2014). Silicon concentrations within specific plant tissues are not always strongly related with transpiration rate, with silicification of silica cells (specific epidermal cells filled with silica) mainly occurring at night (Blackman, 1969) when transpiration rates are low. Silicon deposition has also been found to be independent of water evapotranspiration (Kumar et al., 2016), even when transpiration played a role in the uptake of Si into plants. Further, evidence of silicification of live cells in the absence of transpiration suggests that the cells are actively moving Si into the cells independent of transpiration (Kumar et al., 2016). This may explain the highly organized and distinctive patterns of deposition observed in different species (Hartley et al., 2015).

Increases in Si uptake and changes in Si deposition in response to herbivory may also suggest active redirection of Si within the plant (Hartley et al., 2015). Silicon defenses are now known to be inducible, with up to 400% increases in Si in response to leaf damage (Massey and Hartley, 2006; Massey et al., 2007b). Herbivory-induced increases in Si occur in response to a range of herbivores, persist for several months and have been demonstrated in the field (Massey and Hartley, 2006; Massey et al., 2007b; Garbuzov et al., 2011; Reynolds et al., 2012; Soininen et al., 2013; Hartley et al., 2015; Wieczorek et al., 2015). To date, no studies have tested whether this increase occurs due to leaf damage leading to higher rates of water loss (i.e., increases in transpiration) and thus subsequent changes in uptake and deposition of Si, or if there is an up-regulation in Si transporter genes in the root, brought on by a damage response from the leaves.

Festuca arundinacea Schreb. (tall fescue) has been classified as both a Si accumulator (Hodson et al., 2005) and a non-accumulator (Ma et al., 2001), suggesting its Si uptake in the natural environment is not uniform. Silicon uptake and deposition is relatively uncharacterized within this species, though previous work (Hartley et al., 2015) has shown it has the ability to take up and deposit Si upon the leaf epidermis, and that the levels of Si within the leaf tissues and the structures it enriches differ amongst breeding varieties within the species (very very soft = 0.43% – 0.69% Si, harsh = 0.46% –0.80% Si). Varieties have been described as harsh and soft in terms of their leaf texture, which reflects Si deposition (Hartley et al., 2015). However, how these varieties respond to damage, in terms of Si uptake, and whether any damage-induced increases in Si result from changes in passive Si uptake via transpiration or other more active processes mediated by plant defense responses has not been tested. To date, the studies that have investigated the effects of transpiration on Si uptake have not included an assessment of the effects of damage. Previous studies have focused on the role of transpiration in undamaged

plants in cucumber over a short period of time (Faisal et al., 2012) or in detached leaves placed on solution to understand the silicification of cells within the leaf (Kumar et al., 2016). In contrast, our study investigates the effects of herbivore-simulated damage, in an attempt to understand mechanisms driving the induction process. The aim of this study was to determine if damage-induced increases in Si uptake could be explained by environmental variables such as differences in transpiration rates, or if Si-induced defenses are mediated at gene level by changes in Si transporter expression.

This study investigates how altering transpiration rate and simulating herbivory affects the Si concentration of three varieties of *F. arundinacea*. We hypothesize that:

(1) If Si uptake is largely a passive process associated primarily with transpiration rate, varietal differences in Si concentration will be driven by differences in stomatal conductance and stomatal density;
(2) Damage will induce an increase in Si uptake and varieties with a greater rate of Si uptake and deposition will also show a larger induction response and an increased expression of Si transporters;
(3) If damage-induced increases in Si uptake are driven by changes in water relations, then reducing transpiration differences between undamaged and damaged plants will prevent this increase in Si uptake after damage.

4.2 Materials and Methods

4.2.1 Plant Growth and Experimental Treatments

Three genotypically distinct breeding varieties of *F. arundinacea* contrasting in their ability to accumulate Si (under standard greenhouse conditions, average leaf Si concentrations: very very soft = 0.44%; very soft = 0.43%; and harsh = 0.55%) and varying in leaf texture were provided by the commercial seed company DLF Seeds Ltd., Denmark. The leaf texture is a qualitative trait measured and defined by plant breeders according to how harsh or soft the leaf texture felt on a numerical scale. These were:

- VVS (very very soft leaf texture);
- VS (very soft leaf texture);
- H (harsh leaf texture).

Plants were grown individually in a loam-based compost (John Innes No.2) in 13 cm plastic pots in standard greenhouse conditions: 16 h daylight, 20 °C day, 15 °C night. Once established, plants were randomly subjected to a combination of bagging and damage treatments:

- Undamaged;
- Damaged;
- Undamaged or damaged, then placed in perforated plastic bags.

The aim of the bagging treatment was to control water flow through the plant; bagging the plants would subject both damaged and undamaged plants to similar levels humidity, thus reducing transpiration (Sellin et al., 2014). Treatments were applied four weeks after sowing, with plants harvested 8 weeks later. There were ten replicate plants of each variety per treatment. Plants were watered twice a week with 100ml of deionized water with 150 mgL^{-1} dissolved sodium metasilicate ($Na_2SiO_3 \cdot 9H_2O$); tap water was added as required. In the treatments where damage was applied, half of the total leaves of each plant were damaged twice a week using a metal file. Damaged and undamaged leaves were separated at harvest and leaf Si concentration analyzed separately.

4.2.2 Epidermal Peel Analysis

During the plant harvest, 5 cm of one leaf from eight replicate plants of each variety per treatment were clipped and painted with clear nail varnish. Transparent sticky tape was placed onto the nail varnish once dried, peeled off and the tape stuck to microscope slides. The slides were analyzed via Nikon Eclipse Ni-U light microscope (Nikon Instruments, Kingston Upon Thames, Surrey) for stomatal, trichome, and phytolith counts.

4.2.3 Silicon Analysis by Portable X-Ray Fluorescence Spectrometry (P-XRF)

Si was analyzed by portable P-XRF, calibrated using Si-spiked synthetic methyl cellulose and validated using Certified Reference Materials of NCS DC73349 ‘Bush branches and leaves’ obtained from China National Analysis Center for Iron and Steel. Leaf material was ball milled (Retsch MM 400, Haan, Germany) for 2 min at a vibrational frequency of 30 Hz (60 min^{-1}) with 2 cm diameter steel grinding balls in 25 ml grinding jars. Leaf material was pressed at 10 tons into 13 mm diameter pellets with a manual hydraulic press using a 13 mm die (Specac, Orpington, United Kingdom). Silicon analysis (% Si DW) was performed using a commercial P-XRF instrument (Niton XL3t900 GOLDD analyzer: Thermo Scientific Winchester, United Kingdom) held in a test stand (SmartStand, Thermo Scientific, Winchester, United Kingdom; Reidinger et al., 2012).

4.2.4 Stomatal Conductance Measurements

Stomatal conductance measurements were taken using the Delta –t AP4-UM-3 porometer (Delta-T devices Ltd, Cambridge, United Kingdom). The porometer was calibrated according to the manufacturer’s instructions and then the porometer probe was placed on the leaf and the

time taken for the leaf to release sufficient water vapor to change the relative humidity in a small chamber by a fixed amount was measured; once stabilized (i.e., the same value was observed for two consecutive readings), the stomatal conductance value was recorded. Five readings per variety, per treatment were taken 1 or 2 days after treatments on five different days. Separate readings of undamaged leaves and damaged leaves of damaged plants were taken.

4.2.5 RNAseq and Differential Gene Expression Analysis (DGEA)

At harvest, three biological replicate samples of unbagged, undamaged, and damaged roots for the VVS and H varieties were flash frozen in liquid nitrogen for RNA extraction. RNA was extracted using TRIzol™ Reagent method from 100 mg of root material according to manufacturer's instructions (Invitrogen, United Kingdom). The RNA quality was checked on a 1% agarose gel to test for degradation and quantified using NanoDrop. DNA digestion and cDNA libraries were prepared and sequenced by Leeds Institute of Molecular Medicine (Leeds, United Kingdom). Sequencing was performed using Illumina HiSeq 3000 (Illumina, Inc., United States) using one lane for all libraries, comprising 2 × 150 bp paired end reads. For library assembly, low quality reads and adapter sequences were removed from the raw FASTQ files using Cutadapt[1] with parameters set to: quality >20 and read length >75 bp. The transcriptome was assembled *de novo* using Trinity RNA-Seq 2.1.1 according to the online user-guide[2]. Library reads were aligned to the transcriptome using bowtie2 (Langmead and Salzberg, 2012) and transcript abundance calculated using the RNA-Seq by Expectation Maximization (RSEM) method (Li and Dewey, 2011). Transcripts were annotated in Trinotate v3.0 using BLAST searches (*E* value < 10^{-20}) against Swissprot. DGEA was carried out on the annotated transcripts using the edgeR package (Robinson et al., 2010; McCarthy et al., 2012) to test for differences in log fold changes (logFC) > 1 with a false discovery rate (FDR) set to <0.05 to correct *P*-values for multiple testing. To confirm the identity of Lsi2 sequences, the transcripts were searched for sequence similarity to using BLAST and their transmembrane domains were compared to the barley Lsi2 (accession AB447483.1; Mitani et al., 2009a) sequence using TMHMM Server v2.0[3]. The sequences for *Lsi2* (Supplementary Table 4.1) were only partial sequences, but the transmembrane domains found in these sequences closely matched those in the barley Lsi2 transporter.

[1] https://github.com/marcelm/cutadapt

[2] https://github.com/trinityrnaseq/trinityrnaseq/wiki

[3] http://www.cbs.dtu.dk/services/TMHMM/

4.2.6 Statistical Analyses

All statistical analyses were performed using R (version 3.3.2). Analysis of variance (ANOVA) tests were used to test the main and interactive effects of variety, bagging and damage (using damaged leaves of damaged plants) on leaf Si concentration and stomatal conductance. Paired *t*-tests were used to test for statistical differences between undamaged leaves and damaged leaves of the same damaged plants, where the aim was to test for localized and systemic responses in Si uptake and differences in stomatal conductance. Bonferroni's correction was applied for *t*-tests, setting the level of significance to $P < 0.02$. Generalized linear models were used to test the main effects of variety on stomatal, trichome, and phytolith densities. Linear models were used to check for normality and homogeneity of variance following Crawley (2007). Silicon (%) values were transformed using the arcsine square root transformation to meet the assumptions of the tests. Significance was set at $P < 0.05$ for all analyses other than *t*-tests. Linear regression was used to test for relationships between stomatal conductance and Si concentration. *Post hoc* Tukey tests were carried out and significance was set at $P < 0.05$. Where models did not meet the assumptions, generalized linear models were applied instead of linear models. Packages used for analyses were as follows: lsmeans package (Lenth, 2016), multcompView (Graves et al., 2015), and ggplot function from ggplot2 package (Wickham, 2009).

4.3 Results

4.3.1 Undamaged Plants

Stomatal conductance did not differ significantly between the three varieties but there was a trend for increased stomatal conductance with increasing harshness: VVS displayed the lowest stomatal conductance. Stomatal density, trichome density and Si concentration differed between variety. Stomatal density was higher in the H variety ($F_{2,23} = 4.05$, $P = 0.03$, Figure 4.1 A) compared with the VS variety.

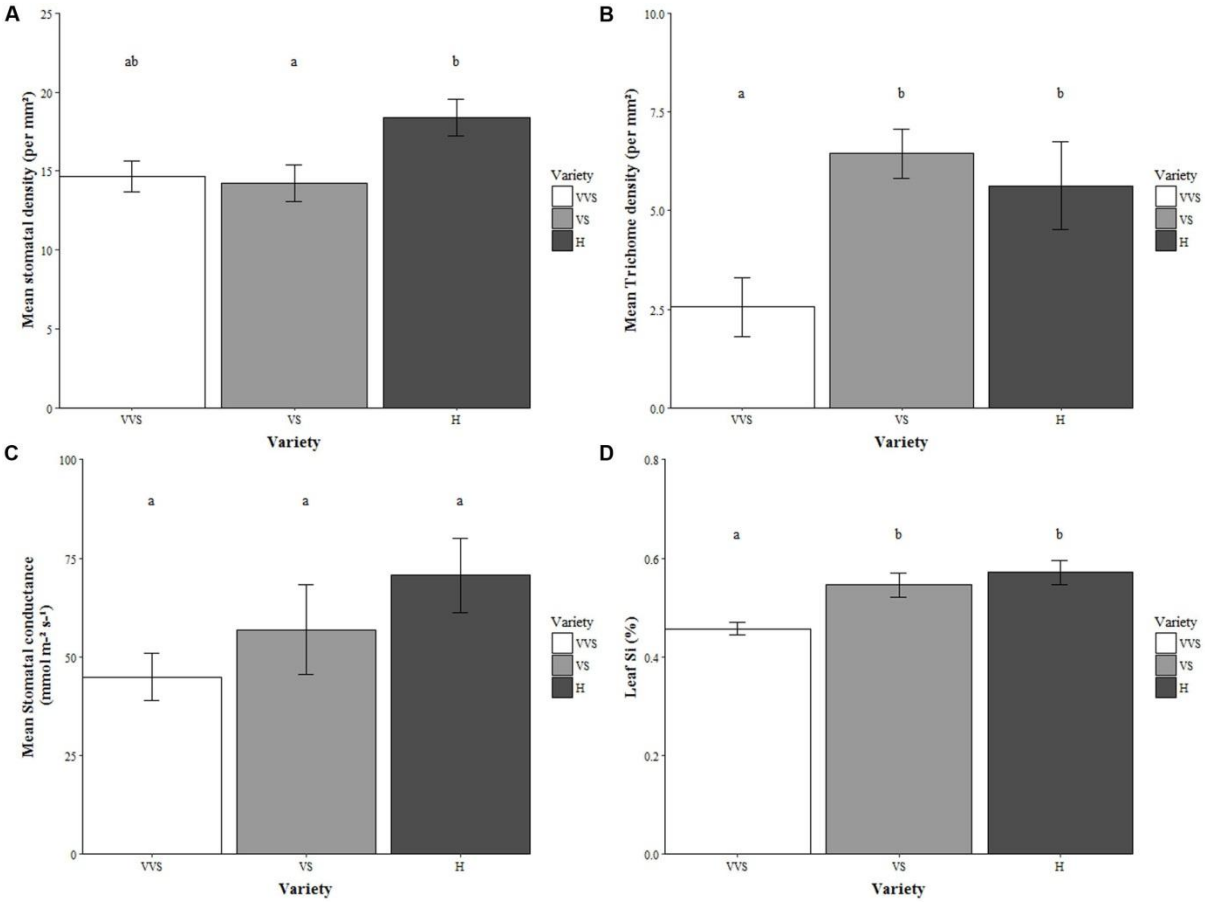

Figure 4.1: (A) Stomatal density, (B) Trichome density, (C) Stomatal conductance, and (D) Leaf Si concentration. VVS = very very soft, VS = very soft, and H = harsh. Values represent unbagged and undamaged plants. Bars are mean values ± SE. $N = 9$ for stomatal density, $n = 5$ stomatal conductance and $n = 10$ for leaf Si. Different letters denote significant differences between treatments (*post hoc* Tukey $p < 0.05$).

The VS and H varieties had more trichomes per mm^2 compared to the VVS variety ($F_{2,23} = 6.02$, $P = 0.008$; Figure 1B), but phytolith density did not differ between the varieties (Supplementary Table 4.2)

The H and VVS varieties differed in their leaf Si concentration ($F_{2,18} = 8.75$, $P = 0.002$; Figure 4.1 D). There was a positive relationship between stomatal conductance and Si concentration ($n = 15$, $r = 0.52$, $P = 0.049$; Figure 4.2 A) in undamaged, unbagged plants.

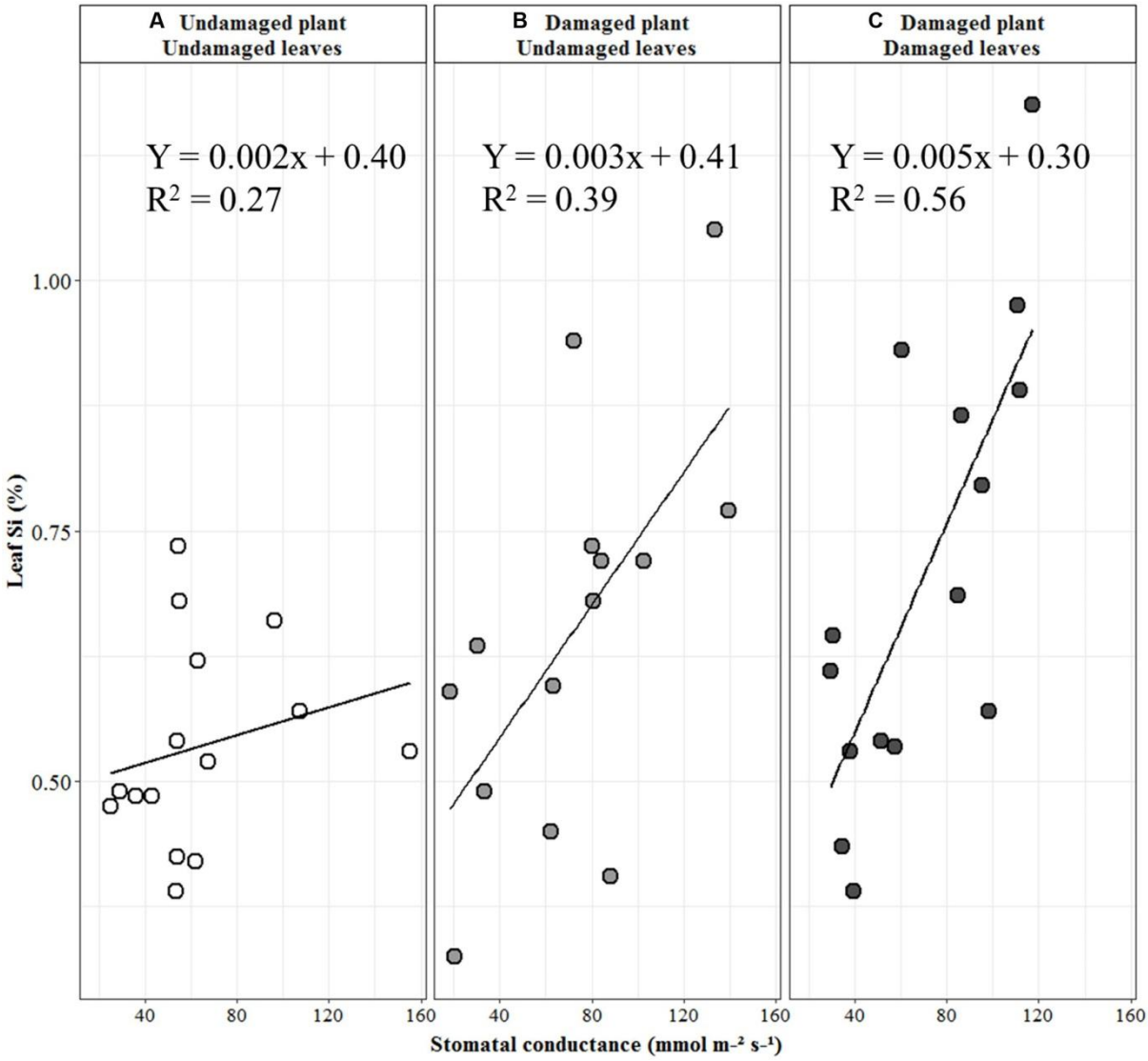

Figure 4.2: Linear regression between stomatal conductance and leaf Si concentration of unbagged plants. (A) Undamaged plants. (B) Damaged plants, undamaged leaves. (C) Damaged plants, damaged leaves. Regression line equation based on raw Si and stomatal conductance data; statistical analysis based on arcsine transformed Si data (see text for details).

4.3.2 Damaged Plants

Stomatal conductance was higher in the damaged leaves of damaged plants in the H variety compared with the VVS variety ($F_{2,12}$ = 6.38, P = 0.01; Figure 4.3 A). There were no differences in stomatal conductance between undamaged leaves and damaged leaves of damaged plants in any of the three varieties.

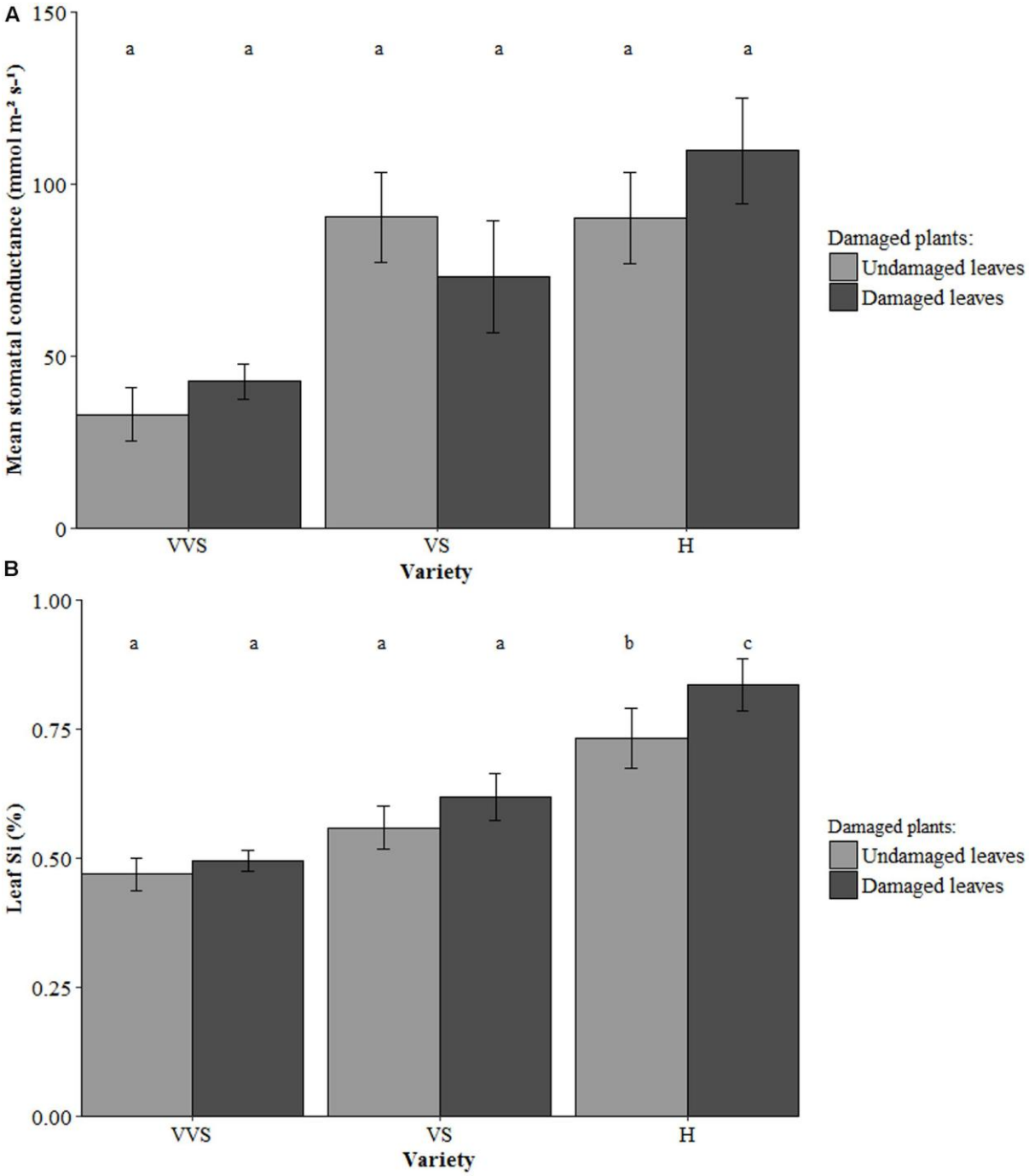

Figure 4.3: (A) Stomatal conductance, and (B) Leaf Si concentration of damaged plants in unbagged conditions. VVS = very very soft, VS = very soft and H = harsh. Bars are mean values ± SE. $N = 5$ for stomatal conductance and $n = 10$ for Si concentration. Different letters denote significant differences between treatments (*post hoc* Tukey $p < 0.05$).

In undamaged leaves of damaged plants, the VVS variety had significantly fewer trichomes per mm^2 compared to the VS and H variety ($F_{2,23} = 5.03$, $P = 0.02$; Supplementary Table 4.2)

In the damaged leaves of damaged plants, no significant varietal differences were observed in terms of trichome density – although there was still a trend for the VVS variety to have fewer trichomes compared to the VS and H variety. Phytolith density was highest in the VS variety for

both undamaged leaves of damaged plants ($F_{2,23}$ = 8.20, P = 0.002) and damaged leaves of damaged plants ($F_{2,23}$ = 813.83, P < 0.001; Supplementary Table 4.2)

The damaged leaves of damaged H plants had more Si than both VS and VVS subjected to this treatment ($F_{2,27}$ = 19.89, P < 0.001; Figure 4.3 B). Paired *t*-tests between undamaged leaves and damaged leaves of damaged plants showed there was a localized response to Si uptake in the H variety only – i.e., the damaged leaves had more Si compared to the undamaged leaves of the same plant (t = 4.58, df = 8, P = 0.002). There was a significant positive linear relationship between leaf Si concentration and stomatal conductance under damaged, unbagged conditions for both undamaged leaves (n = 15, r = 0.62, P = 0.02; Figure 4.2 B) and damaged leaves of damaged plants (n = 15, r = 0.75, P = 0.001; Figure 4.2 C).

4.3.3 RNA-Seq (Unbagged Plants)

The RNA-Seq results revealed five isoforms of *Lsi1* and eight isoforms of *Lsi2* that were expressed in *F. arundinacea*. The Lsi1 isoforms were not differentially expressed between the varieties, nor between the treatments. Out of the eight isoforms found in this species, only three were significantly differentially expressed.

In unbagged, undamaged conditions the H variety had a higher expression of two *Lsi2* gene isoforms compared to the VVS variety in undamaged conditions. A significant increased log fold change from VVS to H of 3.72 was found in TRINITY_DN45085_c2_g1_i1 and a significant increased log fold change from VVS to H of 7.60 in TRINITY_DN45085_c2_g2_i2 in Supplementary Table 4.1).

In unbagged, damaged conditions three *Lsi2* gene isoforms were expressed, and these were up-regulated in the H variety compared to the VVS variety. A significant increased log fold change from VVS to H of 4.52 for TRINITY_DN45085_c1_g1_i1: this isoform was not found to be significantly differentially expressed between VVS and H in the undamaged plants, a significant increased log fold change from VVS to H of 3.51 TRINITY_DN45085_c2_g1_i1 in and a significant increased log fold change from VVS to H of 6.78 in TRINITY_DN45085_c2_g2_i2 (Supplementary Table 4.1 and Figure 4.5).

4.3.4 Bagged Plants

Under bagged conditions, the patterns of stomatal conductance between varieties were similar to those in unbagged conditions. VVS had significantly lower stomatal conductance compared to VS and H varieties ($F_{2,24}$ = 19.07, P < 0.001; data not shown).

The VVS variety had significantly fewer trichomes compared to the VS and H varieties under undamaged, bagged conditions ($F_{2,22}$ = 10.96, P < 0.001). This relationship was the same for both undamaged leaves ($F_{2,22}$ = 10.07, P < 0.001, Supplementary Table 4.3) and damaged leaves of damaged plants ($F_{2,22}$ = 6.39, P = 0.007, Supplementary Table 4.3). Phytolith density was higher in the VS variety compared with H and VVS in undamaged, bagged plants ($F_{2,22}$ = 8.63, P = 0.002, Supplementary Table 4.3) and also in damaged, bagged plants with undamaged leaves ($F_{2,22}$ = 12.43, P < 0.001, Supplementary Table 4.3). The H variety had significantly fewer phytoliths on damaged leaves compared with the other two varieties under damaged, bagged conditions ($F_{2,22}$ = 6.23, P = 0.007, Supplementary Table 4.3).

In bagged conditions, leaf Si concentration did not differ between varieties in either the damaged leaves or undamaged leaves (Figure 4). However, damaged leaves of damaged plants had significantly higher leaf Si than undamaged plants in all three varieties ($F_{1,54}$ = 11.21, P = 0.001; Figure 4.4). No relationship between leaf Si concentration and stomatal conductance was reported for undamaged, bagged plants or for damaged leaves of damaged, bagged plants. There was a weak relationship between leaf Si concentration and stomatal conductance in the undamaged leaves of damaged, bagged plants ($F_{1,13}$ = 0.33, P = 0.03).

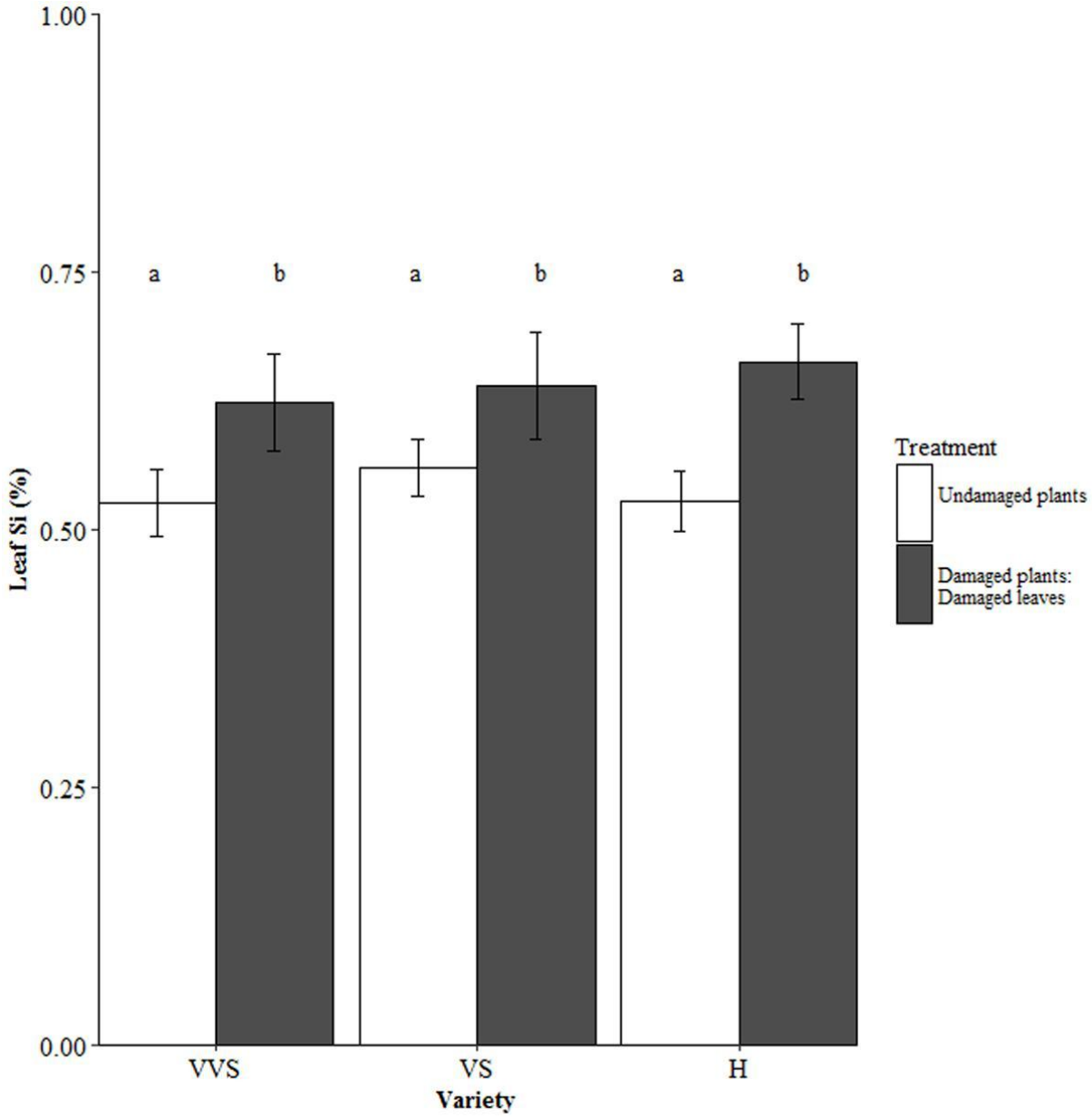

Figure 4.4: Leaf Si concentration of undamaged plants and damaged leaves of damaged plants under bagged conditions. VVS = very very soft, VS = very soft and H = harsh. Bars are mean values ± SE. $N = 10$. Different letters denote significant differences between treatments (*post hoc* Tukey $p < 0.05$).

4.4 Discussion

There are clear differences in the accumulation and deposition of Si between the varieties, and in how the varieties respond to damage in terms of induction of Si defenses. In unbagged conditions, these varietal differences tend to reflect similar differences in stomatal density and stomatal conductance, with the H variety tending to have the highest Si concentration and trichome density as well as the highest stomatal density and stomatal conductance, and with VVS having the lowest. Silicon concentration is significantly positively correlated with stomatal conductance in these plants. The H variety also shows higher induction of Si uptake after

damage than the two soft varieties, increased expression of the active Si transporter *Lsi2* and shows some evidence of systemic induction the other two varieties do not show. However, in bagged conditions, these varietal differences disappear – undamaged bagged plants have the same Si concentration, regardless of variety, and all varieties respond to damage with induction in Si defenses and we no longer see the systemic induction in H plants. Further, these varieties continue to deposit trichomes and phytoliths on the leaf surface under bagged conditions in similar quantities to the unbagged plants, despite the likely difference in transpiration between these two conditions. These findings cannot be explained purely by passive processes linked to water evapotranspiration, implying that damage-inducted increases in Si deposition require active physiologically regulated processes (Kumar et al., 2016).

4.4.1 Undamaged Plants

We hypothesized that if Si uptake was largely a passive process associated with transpiration rate, varietal differences in Si concentration would be driven by differences in stomatal conductance and stomatal density because Si uptake into the tissues, although mediated by the Si transporters, mainly follows the flow of water from the external environment into the root cells (Raven, 1983; Epstein, 1994, 1999; Exley, 2015). Our findings of a correlation between stomatal conductance and Si support this hypothesis of a strong role of the transpiration stream in Si uptake also found in previous studies (Sangster and Parry, 1971; Henriet et al., 2006; Cornelis et al., 2010; Faisal et al., 2012; Kumar et al., 2016) and suggests a strong role of the transpiration stream in Si uptake. However, the clear differences in Si concentration between the varieties, despite no statistical differences observed in stomatal conductance between them, suggests other factors than transpiration stream may have some influence on Si accumulation and deposition in the undamaged plants. The H variety had a higher expression of the active Si transporter *Lsi2* compared to the VVS variety; varietal differences have also been reported in barley cultivars in expression of *Lsi2*, where Si concentration was positively correlated with *Lsi2* expression (Mitani et al., 2009a). It was reported that constant Si supply led to the down-regulation of *Lsi2* in barley and maize over a period of a week (Mitani et al., 2009a). In our study, Si was constantly supplied over a period of 12 weeks, and it is possible that the VVS variety is less able to upregulate *Lsi2* than the H variety under these conditions. These results suggest Lsi2 has an important role in driving varietal differences in terms of Si concentration in tall fescue.

4.4.2 Damaged Plants

We hypothesized that damaging plants would induce an increase in Si uptake, and that varieties with a greater rate of Si uptake and deposition would show larger induction responses. However, in unbagged conditions damaging leaves only elicited a response from the H variety,

both systemically and locally. The undamaged leaves of damaged plants increased leaf Si concentration by 27% and the damaged leaves by 47% compared to the undamaged plants. Such increases in Si after induction have been observed in many other studies (Massey and Hartley, 2006; Massey et al., 2007b; Garbuzov et al., 2011; Reynolds et al., 2012; Soininen et al., 2013; Hartley et al., 2015). Although under undamaged conditions varieties did not differ significantly in stomatal conductance (though there was a trend for higher conductance in harsher varieties), in damaged plants the H variety had significantly higher stomatal conductance than the VVS and VS varieties (see Figures 1C, 3A). This suggests that varietal differences in Si in damaged, unbagged plants may at least in part, be driven by the uptake of water. The lack of response in stomatal conductance, by the VS and VVS varieties is surprising given that most studies (Warrington et al., 1989; Oleksyn et al., 1998; Aldea et al., 2005; Pincebourde et al., 2006) find an increase in stomatal conductance and transpiration when leaves are grazed or perforated, due to damage of the stomata causing impaired function, such as altering the ability of the guard cells to open and close properly. There was also a lack of response to damage in terms of increased Si uptake by VVS and VS varieties, but the VS variety had more phytoliths per mm^2 than in undamaged plants. Thus, although Si concentration did not increase, this variety invested more Si in phytolith production suggesting a shift in allocation patterns of Si under damaged conditions. In damaged plants, there was a greater expression of *Lsi2* gene isoforms compared to the undamaged plants suggesting that this transporter is at least partially responsible for Si-induced defenses in this species. The *Lsi2* transporters were up-regulated in the H variety compared to the VVS variety. Tall fescue is an outbreeding, allohexaploid (Gibson and Newman, 2001) and therefore there may be splice variants of these Si transporters in the different varieties which are only activated upon damage. We were able to see differences between treatments using a small number biological replicates in a species with a complex genome such as tall fescue, providing clear evidence that Si-induced defenses are under molecular control in this species. In barley, Si concentration was positively correlated with *Lsi2* expression (Mitani et al., 2009a), here we also see plants with more Si in the leaves also have a higher expression of *Lsi2*.

4.4.3 Bagged, Undamaged, and Damaged Plants

We hypothesized that if damage induced increases in Si uptake were driven by changes in water relations, bagging plants would prevent this increase in Si uptake after damage. Bagging the plants removed the differences observed between the undamaged and damaged plants in terms of stomatal conductance compared to when plants were not bagged, and also removed the varietal Si differences observed in unbagged plants. However, bagging plants did not remove the Si differences between the undamaged and damaged plants: there was still an induction response to damage, increasing the leaf Si concentration in damaged plants compared to the undamaged plants in all 3 varieties. The systemic induction in the H variety observed in

unbagged damaged plants was not found in this treatment, suggesting systemic induction is in part influenced by water relations, but localized responses to damage with increased Si deposition are not. The trend in trichome and phytolith deposition between the varieties remains similar between unbagged and bagged conditions (i.e., that VVS has less trichomes compared to the VS and H variety and that the VS variety has more phytoliths compared with the H and VVS varieties), again suggesting this deposition is not primarily transpiration driven. We also see differences between the varieties in terms of the deposition patterns, even though the stomatal conductance is the same (Supplementary Table 4.3). Transpiration seems necessary for plants to accumulate Si from the roots to the leaf tissues, but other active means must be at play during the deposition to explain findings in our study. Other work supports this assertion, silica accumulation in silica cells takes place only during leaf development (Sangster, 1970; Motomura et al., 2006; Kumar et al., 2016); if transpiration were the sole cause of Si deposition then all leaves (despite their age) would continue to deposit Si in the silica cells, but this is not the case (Kumar et al., 2016).

Studies that have investigated the relationship between passive/ active uptake of Si in plants have found content of Si both higher (Faisal et al., 2012; Gocke et al., 2013; Kumar et al., 2016) and lower Si than expected for passive uptake (Cornelis et al., 2010), which again goes against the suggestion that Si uptake and distribution is a purely passive process (Exley, 2015). Silicic acid may move freely into the roots but uptake and distribution of Si increases in the presence of the influx and efflux transporters (Ma and Yamaji, 2008; Farooq and Dietz, 2015). Many studies have shown Si transporters are responsible for the uptake and distribution of Si in different grass species (Ma et al., 2006; Chiba et al., 2009; Mitani et al., 2009a,b; Montpetit et al., 2012). Silicon transport, both within and between species is variable as is the regulation of the Si transporters – *Lsi1* is down regulated in rice during constant Si supply after 3 days (Ma et al., 2006) whereas in barley and maize for example, the expression is constitutive (Chiba et al., 2009; Mitani et al., 2009a). In terms of inducible plant defenses, plants may only up-regulate expression of Si transporters as needed and rely on their base transcript levels of Si transporters and transpiration to utilize Si under undamaged, unbagged conditions. Complex interactions between genetic and environmental controls on the expression of transporters may explain why Si levels for the same species are often so variable (Ma et al., 2001; Hodson et al., 2005; Soininen et al., 2013).

Given that Si transporters have been identified in many other species of grass such as rice (Ma et al., 2006) and barley (Mitani et al., 2009a and in some dicotyledons, cucumber, pumpkin, and soybean (Deshmukh et al., 2013) for example (see Deshmukh et al., 2015; Deshmukh and Bélanger, 2016 for others), it is likely that *F. arundinacea* has Si transporters and that differences in these underlie differences in Si uptake and deposition we observe between varieties. Other studies have found intraspecific differences in uptake abilities in rice (Wu et al.,

2006; Ma et al., 2007a) which revealed that the higher Si accumulating genotypes were able to accumulate more Si due to a higher level upregulation of Si transporters. Perhaps this is also the case for the differences in these varieties and may also be why the high accumulating variety (H) is better able to respond to damage as there is a greater number of Si transporters present. The spacing between the conserved (asparagine-proline-alanine (NPA)) domains in Si transporters is also likely to influence uptake abilities within and between species; the spacing between these amino acids have been shown to determine whether plants are able to import or reject importing Si into the root cells (Deshmukh et al., 2015).

4.5 Conclusion

Few studies have looked at the relationship between Si accumulation and transpiration, and to date none have looked at these in combination with damage. To date, no studies have looked at differential expression of the Si transporters between undamaged and damaged conditions to test for molecular evidence of Si-induced responses. There were clear differences in the response of the three varieties to the damage treatments within this study, suggesting that damage is an important driver in the accumulation of Si. Removal of differences in stomatal conductance also removed the difference in Si levels between the varieties, suggesting that transpiration has a role in Si accumulation, but the higher Si levels under damaged, bagged conditions show these increases must occur by mechanisms other than just passive movement of Si in the transpiration stream. This gives clear evidence for active Si-induced defenses within this species. Further, we provide the first evidence of molecular based Si-induced defenses by the up-regulation of the active Si transporter, *Lsi2*, in damaged plants. Clearly, further molecular characterization of the mechanisms involved in Si uptake and transport following damage is necessary to fully understand how Si gets from the xylem and into the cells in the leaves. These results not only provide evidence for Si-defenses being regulated at gene level, they also provide insights into target traits for selecting plant genotypes resistant to herbivory for agriculture and other uses.

4.6 Supplementary Data

Table 4.1: Isoform sequences and percentage identity to known Lsi2 transporter sequences. N.B. ArsB is a group of efflux transporter proteins and Lsi2 belongs to this group.

Isoform	Transcript Sequence	BLAST % Identity
TRINITY_DN45085_c1_g1_i1	GAGGTTCTGCGGGTTGCCGATGGGCGTGGCGGAGGAGCCGATGTTGGA GCTGGAGGCGAGGGCGAGGAGGAATGGCTGCGGCGGCAGGTTGTTCTG CCTGGCCACCTTGAGGATGAACTCGGTGAGGACGACGCAGGTGGTGTC GTTGGTGAAGAGCGCGCTGGCGACGGCGGAGACGAGGCAGACGCGGA AGAGGAGGTCCTTGCTGCCGCGGCTCTTCCAGGAGAGCATGCTGCCGA GGTACTGGAACATGTCGGCGCGCTCCAGGAAGATGCTGACCACCATGG TGCCGAAGAGGAGGCCCAGGATGGGGAGGTCGATCGCGGCGTAGGCCT CCTCGGGGGTCATGACGCGGAAGAGCACCATGAGCATGGCTCCCAGC AGAGACCCCGCCGTCCGGCCCACCGGCAGGAACGGCACCGACGGGAA GACGGCCAGCACCCAGAAGATGACGAAGGCGACGCATCCCAGCACCA CCTTGGGCGTGCTCGCCAGCACCATCTCGGATCCTCGGAAGTAAGCAG CACTTGGCAATGAAGAAAGAACTTCAGTTGAGCACAAACTGATCAGC GACAACAATGCCAGTCGATTCGTGTCCACAACGAGGGGCCGTCTACA GGTCTACTCGATCAAGATCCTGCAAAACCGGAGCAAGGAAACAGAAG CTCGAGGGCGGCGGCGGCGAGGGGCTGTCCAGTACCGGGAGAAAGGGC AGACCGGCGGAGAGGTTTGAGCAGGCCTGAACTCACTACATAGTCGG ACGGCAGCTCCACGAGAGTAGGAGTAGGGTAGTGGCGACTGACGATC GAGAGGCAGGGAGGTCGCGTCGTGTTATCCCCCCAGGTTGGGACTTGC GCTACTACTCTACATAGGGGTATTTATTAGGGGCGGTGCAGCCGTGTA AGTTTTCGGAATGGATAGCTAACGGGGAGCTGGTCTGTGTTTGGCTACC GTCAGCAGTAGCTCTGGTTCGATCAGTCTAAGGCACGCCGCACACATG	99% identity sequence similarity to predicted arsB transporter (i.e. Lsi2) in *Brachypodium distachyon*
TRINITY_DN45085_c2_g1_i1	CGACGCCAGGGCCAGCAGGAACGGCTTCGGCGGCAGGTTGTTCTGGCG GGCGATCTTGAGGATGAACTCGGTGAGCACGACGCAGCAGGTGTCGTT GGTGAAGAGCGCCGACGCGAGCGCGGAGACGACGCAGGTGCGGACGA GGAGGTCCTTGCCGCCCTGCGACCGCCACGAGAGGAGCCGGCCGAGGT GCCGGAACATGTCGGCGCGCTCCAGGTACACGCTCACCACCATGGTGC CGAAGAGCAGGCCCAGGATGGGGAGGTCCACGGCCGCGTAAGCCTCG TCGGCGGTGATGACGTTGAAGAGCACCATGAGCATGGCGCCCAGGAGC GACCCCGCCGTCCGGCCGATAGGCAGGAACGGCACCGCCGGGAACAC CGCCAGTACCCAGAAGATGGCGAATGACGCCGTGCCCAGTGCCACCTT CACCGTCGGCTCCATCGCCATTGCTGAATTGCCTACTTCAGCTCAAGC TAAAGATTCGACGTCAACACTGAATCTGCGGGAATCACCTCTTGGATA TACCACCTCTCGGGATTCAATCCAAGAACACCGAGCTACCTAACTCTT CACCGATAAATCTAGAAACTAAATTCTGCAATCTAGCTACAAGATCT GTGCCTTGCTGCTCACTGGACCAGCTAGCTCACATGGGAAGCTACCAA CAATAACAAGAGCCGGACCTCAACTGCAATTCCAATCAGCTAATGGG GGCACACAGCCATGCACGAACACGTACCAATAAACCACCAATTTGAT CAGGATGCCTCCATGGATCCCAAGGAAACCAGTCTACGAAGGTTGAA GAAGATGATGATGAAGAGCTTGTGCAGGAACTACCTAGCAGCTAGTA GTGGATGTCGCGACATGGCATGACATGTGGTTCTTCGGAGGAGGAATG TAACACTTGGCATGCTCCACATGGCCTCCTAGTAGATTGCCCCACCTG GATATTATTTTCTCTCTACCTCTCTACCAATCGATCGATGGGACAATGT CC	98% Identity sequence similarity to predicted protein ArsB (i.e. Lsi2) *Hordeum vulgare* subsp. v*ulgare*
TRINITY_DN45085_c2_g2_i2	GGGCAGGTGTAATATGAAAGTATTGCAACATATATAGTTGGATCGGCC ACAGCGGGCGGCGGTGAGGTCTACATCTTTCCGATGAGGGGGATGCCA ACGGCGGTGACGATGAGAGTGGAGGGAACACCGAAGATGAGGTGGTT CCAGAAGGTGAGTTCATACGCGTTACGTGGCGCCCGGCGCGCTTGCTC ACAGACGATGAGGTTCGCCGCCGACCCTAGTAGCGACAGGTTCCCCGC CACCGTGCTCACCCACGCCAGCAGTAGCCATGACCGAGTCACTGCTGC CGGGGAGATCAAAGCCGCGGCGGTCGCCACCTCGTTCCCCATCAGAAG CACTGTTGGTACGTTGGAGGCGAGGTTGGAGAGGAGGAGGATGATGAT GGAGAGGACGGAGATGCCGCCGGCGCTGTCGACCTTGGAGTAGGGCGC CATGA	99% similarity to Lsi2 in *Hordeum vulgare* (Ma et al. 2009)

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TRINITY_DN45085_c1_g1_i1      ATGGTGCTGGCGAGCACGCCCAAGGTGGTGCTGGGATGCGTCGCCTTCGTCATCTTCTGG
TRINITY_DN45085_C2_G1_I1      ATGGCGATGGAGCCGACGGTGAAGGTGGCACTGGGCACGGCGTCATTCGCCATCTTCTGG
TRINITY_DN45085_c2_g2_i2      ------------------------------------------------------------


TRINITY_DN45085_c1_g1_i1      GTGCTGGCCGTCTTCCCGTCGGTGCCGTTCCTGCCGGTGGGCCGGACGGCGGGGTCTCTG
TRINITY_DN45085_C2_G1_I1      GTACTGGCGGTGTTCCCGGCGGTGCCGTTCCTGCCTATCGGCCGGACGGCGGGGTCGCTC
TRINITY_DN45085_c2_g2_i2      ------------------------------------------------------------


TRINITY_DN45085_c1_g1_i1      CTGGGAGCCATGCTCATGGTGCTCTTCCGCGTCATGACCCCCGAGGAGGCCTACGCCGCG
TRINITY_DN45085_C2_G1_I1      CTGGGCGCCATGCTCATGGTGCTCTTCAACGTCATCACCGCCGACGAGGCTTACGCGGCC
TRINITY_DN45085_c2_g2_i2      ------------------------------------------------------------


TRINITY_DN45085_c1_g1_i1      ATCGACCTCCCCATCCTGGGCCTCCTCT----TCGGCACCATGGTGGTCAGCATCTTCCT
TRINITY_DN45085_C2_G1_I1      GTGGACCTCCCCATCCTGGGCCTGCTCT----TCGGCACCATGGTGGTGAGCGTGTACCT
TRINITY_DN45085_c2_g2_i2      ------------GCCTGGACCTACACCTTCATCCGCTTCGAGGAC------CC-GCCCA
                                         *****.*** *:*     ** **: *.:**:           **:

TRINITY_DN45085_c1_g1_i1      GGAGCGCGCCGACATG----------TTCCAGTACCTCGGCAGCATGCTCT-CCTGGAAG
TRINITY_DN45085_C2_G1_I1      GGAGCGCGCCGACATG----------TTCCGGCACCTCGGCCGGCTCCTC-TCGTGGCGG
TRINITY_DN45085_c2_g2_i2      AGGACGCGCCGCAGAAGCTCTCCTCCTTCAAGCT---CCGCCGGCTGCAGAGCCAGTCCG
                              .*..*******...:.          ***..* :   * **.* .* *:   * :* . *

TRINITY_DN45085_c1_g1_i1      AGCCGCGGCAGCAAGGACCTCCTCTTCCGCGTCTGCCTCGTCTCCGCCGTCGCCAGCGCG
TRINITY_DN45085_C2_G1_I1      TCGCAGGGCGGCAAGGACCTCCTCGTCCGCACCTGCGTCGTCTCCGCGCTCGCGTCGGCG
TRINITY_DN45085_c2_g2_i2      TCGC-----------------CGCAACCG-----ACCTTGACGACGAGCTCGA------A
                              :  *                 * * :***     .* * *:* .**.  ***.      .

TRINITY_DN45085_c1_g1_i1      CTCTTCACCAACGACACCACCTGCGTCGTCCTCACCGAGTTCATCCTCAAGGTGGCCAGG
TRINITY_DN45085_C2_G1_I1      CTCTTCACCAACGACACCTGCTGCGTCGTGCTCACCGAGTTCATCCTCAAGATCGCCCGC
TRINITY_DN45085_c2_g2_i2      CACATCCCCA----------------------------------------------------
                              *:*:**.***

TRINITY_DN45085_c1_g1_i1      CAGAACAACCTGCCGCCGCAGCCATTCCTCCTCGCCCTCGCCTCCAGCTCCAACATCGGC
TRINITY_DN45085_C2_G1_I1      CAGAACAACCTGCCGCCGAAGCCGTTCCTGCTGGCCCTGGCGTCG---------------
TRINITY_DN45085_c2_g2_i2      --------------------------TCTGATCGCCGTTGCGTCGTCGTCGCT----AG-
                                                       ** .* *** * ** **

TRINITY_DN45085_c1_g1_i1      TCCTCCGCCACGCCCATCGGCAACCCGCAGAACCTC
TRINITY_DN45085_C2_G1_I1      -----------------------------------
TRINITY_DN45085_c2_g2_i2      -----------------------------------
```

Figure 4.5: Multiple sequence alignment of differentially expressed *Lsi2* isoforms in unbagged H and VVS plants.

Table 4.2: Trichome and phytolith density of plants under unbagged condition. VVS=very very soft, VS=very soft and H=harsh

UNBAGGED						
Treatment	Trichomes per mm^2			Phytoliths per mm^2		
	VVS	VS	H	VVS	VS	H
Undamaged plant	3	6	6	16	21	15
Undamaged leaves, damaged plant	3	5	6	14	23	13
Damaged leaves, damaged plant	3	4	5	13	23	13

Table 4.3: Trichome and phytolith density of plants under bagged conditions. VVS=very very soft, VS=very soft and H=harsh

BAGGED						
Treatment	Trichomes per mm^2			Phytoliths per mm^2		
	VVS	VS	H	VVS	VS	H
Undamaged plant	1	4	5	16	25	13
Undamaged leaves, damaged plant	1	4	4	13	19	10
Damaged leaves, damaged plant	1	4	3	16	18	11

5 Chapter 5: Differences in Si transporter expression in tall fescue

5.1 Introduction

Silicon (Si) is an element beneficial to plants. It is accumulated by plants in varying concentrations, though leaf Si concentrations rarely exceed 10% dry weight (Ma et al., 2006). Plants have been found to use Si to alleviate both abiotic (Kim et al., 2002, 2014; Pontigo et al., 2017) and biotic stresses (Fauteux et al., 2005; Massey and Hartley, 2006; Massey et al., 2007a,b). Silicon is taken up from the soil in the form of silicic acid (Deshmukh et al., 2013) by Si transporters in the root and translocated to the shoot, where it is deposited on the leaf surface as phytoliths (solid, SiO_2 bodies).

The transporters involved in root Si transportation, Lsi1 and Lsi2, work cooperatively to transport Si from the external environment (i.e. outside of the root), through root cells (symplastically) and released to the xylem (Ma et al., 2006; Ma et al., 2007a). Lsi1 is a passive Si transporter, belonging to the nodulin 26-like intrinsic proteins (NIPs) subfamily, of the major intrinsic protein (MIPs) family (Ma et al., 2006; Deshmukh et al., 2015; Deshmukh and Bélanger et al., 2016). Proteins that belong to this family are aquaporins, and therefore able to transport water and other solutes across biological membranes (Chrispeels and Maurel, 1994; Kruse et al., 2006; Deshmukh et al., 2015). Lsi1 is classified in the group NIP III, based on the presence of a similar aromatic/arginine (Ar/R) filter (Deshmukh et al., 2013; Deshmakh and Bélanger, 2016) and as an aquaporin, it has six transmembrane domains (Maurel et al., 2008). Another defining feature of a NIP III transporter is the presence of 2 highly conserved NPA motifs (Ma et al., 2006; Deshmakh and Bélanger, 2016), with high Si accumulators known to have a precise spacing of 108 amino acids between 2 NPA motifs. *Lsi1* genes have been found in a number of species: rice (Ma et al., 2006), barley (Chiba et al., 2009), maize (Mitani et al., 2009b), soybean (Deshmukh et al., 2013) and strawberry (Ouellette et al., 2017) to name a few, with most of studies focussing on monocotyledons. Lsi2 is an efflux transporter and was first characterised in rice, it is highly expressed in mature regions of the root, with very low expression in the root tips (Ma et al., 2007a). Silicon efflux transporters are characterised by having 9 -12 transmembrane domains (Deshmakh and Bélanger, 2016) and are localised in the plasma membrane (Sakurai et al., 2015), though much less is known about this putative family of proteins. Lsi2 is thought to be actively involved in the transport of Si through the use of a proton antiporter (Ma et al., 2007a; Ma et al., 2011; Sakurai et al., 2015).

Silicon is known for being involved in both constitutive plant defense and inducible defenses (Massey and Hartley, 2006; Ye et al., 2013; Frew et al., 2016). However, the exact mechanisms

behind inducible Si-based defenses remain unknown. Many studies report increases in Si in response to herbivory (McNaughton and Tarrants, 1983; Massey and Hartley, 2006; Ye et al., 2013; Wieczoreck et al., 2015). Molecular studies involving Si transporter expression and herbivory are lacking and few have looked into intraspecific differences in Si transporter expression. A recent study has looked into the molecular mechanisms behind Si transporter expression in rice when exposed to the rice leaf folder caterpillar (Ye et al., 2013). This study found an upregulation in the expression of *Lsi2* in wild type plants when Si had been added in the presence of a herbivore, compared to the plants without herbivory. However, further evidence is required to fully understand the mechanisms involved in inducible Si-based defenses, and whether leaf Si concentration post-herbivory can be explained by increased expression of the Si transporters.

Festuca arundinacea Schreb. (tall fescue) is a forage and turf grass, grown widely over the world (Hand et al., 2012a). Tall fescue is an outbreeding, allohexaploid (Hand et al., 2010) and as such, little molecular work has been done on the species. However, as this grass is widely grown for animal forage and turf, understanding important agronomic traits is beneficial for plant breeders. Tall fescue is a Si accumulator (Hodson et al., 2005; Hartley et al., 2015; McLarnon et al., 2017) and recently a number of potential isoforms of *Lsi2* were identified in this species (McLarnon et al., 2017). *Lsi2* expression was higher in a variety described as harsh (in terms of leaf texture) and these plants accumulated more Si in response to leaf damage. However, a number of other isoforms of *Lsi1* and *Lsi2* (data not shown) were also found. Using these Si transporter sequences, this study aimed to further investigate the expression of Si transporters in tall fescue varieties, looking at a range of harsh and soft plants, to understand the nature of inducible Si-based defenses in this species. Specifically, this study aimed to determine if varieties described as harsh and soft have different expression levels of Si transporters, and if these may be partly responsible for the difference in leaf Si deposition observed in previous work. The following hypotheses were tested:

1. Damaged plants will take up more Si than undamaged plants, which will be mediated by an upregulation of Si transporter expression in response to damage.
2. Varieties described as harsh will take up more Si compared to soft varieties, and Si transporter expression will be greater in harsh varieties compared to soft varieties.

5.2 Methods

5.2.1 Plant growth and varieties used

Eight varieties of tall fescue separated into two groups of harshness: 4 soft and 4 harsh varieties (as defined by plant breeders, according to a qualitative leaf texture scale). These varieties were provided by DLF Seeds and were scored discretely on the harsh or soft varieties. A number of harsh and soft varieties (Soft: DLF-04-D (D), DLF-05-J (J), DLF-06-8 (8), DLF-07-F (F) and harsh: DLF-10-M ((M) chapter 2), DLF-11-C (C), DLF-12-T (T), DLF-13-K (K), DLF-14-N (N)) were selected to expand on the molecular work carried out in chapter 4, with the aim to understand if damaged-induced changes in Si transporter transcripts were specific to all harsh varieties and to understand if these changes were driving the harsh leaf texture.

Plants were grown in standard greenhouse conditions: 16 h daylight, 20 °C day and 15 °C night in a complete randomised block design. Plants were watered twice weekly with 100 ml of 150 mgL^{-1} sodium metasilicate ($Na_2SiO_3.9H_2O$) dissolved in deionised water. Additional deionised water was added as required. After establishment, the plants were randomly assigned to two treatments: no damage or damage, which was applied twice a week, 4 weeks after the seeds were sown, for 15 weeks. Damage was carried out using a single hole punch to remove half of the leaf tissue, this was applied to half of the total leaves per plant, resulting in damage levels of 25% to simulate realistic levels of insect herbivory. Plants were harvested 19 weeks after the seeds were sown.

5.2.2 Silicon analysis

Si was analyzed by portable-X-ray florescence (P-XRF), calibrated using Si-spiked synthetic methyl cellulose and validated using Certified Reference Materials of NCS DC73349 ‘Bush branches and leaves’ obtained from China National Analysis Center for Iron and Steel. Leaf material was milled (Retsch MM 400, Haan, Germany) for 2 minutes at a vibrational frequency of 30 Hz (60 min^{-1}).Leaf material was pressed at 10 tons into 13mm diameter pellets with a manual hydraulic press using a 13 mm die (Specac, Orpington, UK). Silicon analysis (% Si DW) was performed using a commercial P-XRF instrument (Niton XL3t900 GOLDD analyzer: Thermo Scientific Winchester, UK) held in a test stand (SmartStand, Thermo Scientific, Winchester, UK; Reidinger et al., 2012).

5.2.3 Primer design and PCR

Sequences annotated as Lsi1 and Lsi2 (from RNA-Seq data in Chapter 4) were aligned to known Si transporter sequences. Top hits from BLAST results were used to detect the correct frame, in order to obtain the correct start codon. Primer3 design software (http://primer3.ut.ee/) was used to design primers for transcripts annotated as Lsi1, Lsi2 and eukaryotic elongation

factor (EeF1) including the three isoforms which were reported to be differentially expressed when plants were damaged (McLarnon et al., 2017) for real time-quantitative polymerase chain reaction (RT-qPCR) (Appendix 1 and Table 5.1). Three primer pairs per sequence were tested using previously prepared tall fescue cDNA and DreamTaq Green DNA Polymerase (5 U/µl) for product amplification. Product amplification and size were determined by gel electrophoresis using a 3% agarose gel. Primers that successfully amplified a product of the correct size were used to clone the PCR products.

Table 5.1: Primer conditions for each primer pair for RT-qPCR

Identifier	Annotation	Orientation	Primer Sequence 5'-3'	Primer size (nts)	Amplicon size (nts)	Tm	GC content (%)
TRINITY_DN41992_C1_G2_I1	Lsi1	Forward	GCCTGGACCTACACCTTCAT	20	144	59.09	55
TRINITY_DN41992_C1_G2_I1	Lsi1	Reverse	GAGCTTGAAGGAGGAGAGCT	20		58.81	55
TRINITY_DN45085_C2_G2_I1	Lsi2	Forward	CATGGTGGTCGCATACATGG	20	141	59.05	55
TRINITY_DN45085_C2_G2_I1	Lsi2	Reverse	CCGGAAAAGAAGACGAGCAG	20		58.93	55
TRINITY_DN28909_c0_g1_i1	eEF1	Forward	CACAGTCATTGATGCCCCTG	20	119	58.9	55
TRINITY_DN28909_c0_g1_i1	eEF1	Reverse	TACCAGCCTCAAAACCACCA	20		59.15	50

5.2.4 Cloning, transformation and plasmid prep

Cloning was carried out using StrataClone PCR cloning kit (Agilent Technologies, USA) according to the manufacturer's instructions: the ligation reaction mixture contained 3 µl StrataClone cloning buffer, 2 µl PCR product, 1 µl StrataClone Vector mix (amp/kan). This reaction was mixed, incubated for 5 minutes at room temperature and placed on ice. StrataClone SoloPack competent cells were thawed on ice and 1µl of ligation mixture was added, incubated on ice for 20 minutes and then heat shocked at 42 °C for 45 seconds. The samples were then incubated on ice for 2 minutes before 250 µl of Luria broth (LB) medium was added. The samples were then placed on shaker for 2 hours at 37 °C to enable the competent cells to recover and 50 µl of this transformation mixture was spread onto LB agar carbenicillin plates containing 40 µl of 2% X-gal, to enable blue-white colour screening, and left to incubate for 16 hours at 37 °C. White colonies (approximately 8-16 per primer pair) were selected and colony PCR was carried out on each colony using universal M13 primers and DreamTaq Green DNA polymerase. The PCR products were tested to ensure the plasmids contained the PCR products uptake by gel electrophoresis using a 1.5% agarose gel. Plasmid DNA was purified using the Wizard® *Plus SV* Miniprep DNA purification kit (Promega, UK) according to the manufacturer's instructions: 4ml of LB broth with carbenicillin and 20 µl of RNase-free H_2O containing transformed cells (obtained by washing the pipette tip used to transfer colonies to fresh agar plates for overnight growth) were shaken overnight for 16 hours at 37 °C. These tubes were centrifuged and supernatant discarded, with the pellet resuspended in cell resuspension solution and lysed using cell lysis solution. Alkaline protease solution was added, the mixture was neutralised by neutralising solution and then centrifuged. The lysate was then washed and plasmid DNA was eluted and stored at -20 °C.

5.2.5 Plasmid digestion and sequencing

Plasmid DNA was digested to verify size of inserted amplicon and to determine that amplicons of the correct size were taken up during cloning. Digests were carried out using EcoRI restriction enzyme and product size was determined by gel electrophoresis using a 2% agarose gel. Plasmids carrying PCR products that were of the expected amplicon size (50-150 bp) were sent for Sanger sequencing at GATC Biotech (GATC Biotech AG, Germany). The sequencing results were put through BLAST to ensure the primers amplified the amplicons from the Si transporters of interest. Primer sequences which amplified the correct Si transporter amplicons were used for RT-qPCR.

5.2.6 Silicon transporter sequences and alignments

Full length sequences of transporters selected for RT-qPCR analysis (Appendix 1) were aligned (using the nucleotide sequences obtained from RNA-Seq, chapter 4) to the top BLAST hit(s). Nucleotide sequences were converted into protein sequences using the ExPASy Translate tool[4] and the open reading frame from the top BLAST hit was used to determine the protein sequence. Alignments for Lsi1 were done using MEGA (version 7), alignment option clustalW to detect the NPA motifs. Transmembrane domain sequences (TMD) of the proteins were determined using TMHMM Server v2.0[5]. The conserved domains were confirmed using CDD database[6]. SWISS-MODEL[7] with the default settings applied, was used to predict the 3D structure of the predicted Lsi2 proteins for barely and tall fescue.

5.2.7 RNA extraction and cDNA synthesis

Three replicate root samples of each variety, of undamaged and damaged plants were taken (N = 48) at harvest and flash frozen in liquid nitrogen. These samples were stored at -80 °C. The RNA extraction was carried out using Direct-zol™ RNA MiniPrep (Zymo Research, Canada) according to the manufacturer's instructions using 100 mg of root material homogenised in liquid nitrogen for each sample. The optional in-column DNase digest was carried out. The RNA was quantified using Nanodrop and the quality tested by gel electrophoresis using a 1% agarose gel, to check for degradation and contamination. The cDNA was prepared using SuperScript II RT (Invitrogen™, UK) according to manufacturer's instructions using oligo dT primers and 1 µg of DNased RNA. The cDNA samples were stored at -20 °C.

5.2.8 RT-qPCR

Primers for previously annotated Lsi1, Lsi2 and eEF1A (as a housekeeping reference gene) were designed (Appendix 1 and Table 5.1) as described above (sequences were obtained from RNA-Seq data, chapter 4). Reverse transcriptase quantitative PCR (RT-qPCR) was performed in 96 well plates using Fast Syber Green Master Mix in a total volume of 20 µl (Thermo Fisher Scientific, UK). Standard curves to determine primer efficiencies for housekeeping gene and Si transporters were carried out using serial dilutions of cDNA at 5 different concentrations. For each 20 µl reaction, 3 µl of cDNA used and primer final concentration of 0.3 µM, cDNA samples were carried out in duplicate and one plate per primer pair was used. The analysis was carried out on Applied Biosystems QuantStudio™ 3 Real Time PCR system (Thermo Fisher Scientific, UK) with the default machine settings for RT-qPCR. The programme cycles were as follows: Hold stage: 1 cycle of 95 °C for 20s, PCR stage: 40 cycles of 95 °C for 1s and 60 °C for 20s, melt curve stage: 1 cycle of 95 °C for 1s, 60 °C for 20s and 95 °C for 1s. A final

[4] (http://web.expasy.org/translate/)
[5] (http://www.cbs.dtu.dk/services/TMHMM/)
[6] (https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml)
[7] (https://swissmodel.expasy.org/interactive)

denaturation step of the PCR products was included in the program to determine the primer specificity by checking for single product amplifications. Silicon transporter expression was normalised using the housekeeping gene eukaryotic elongation factor 1 (eEF1A) and relative changes in gene expression were carried out using primer efficiencies according to Pfaffl, (2001): (primer efficiency target $^{-Ct\ target}$)/ (primer efficiency reference $^{-Ct\ reference}$), where the reference gene was eEF1A. These values were used to test for differences in expression of Lsi1 and Lsi2 between undamaged and damaged plants (method adapted from Soukup et al., 2017).

5.2.9 Statistical analysis

All statistical analyses were carried out in R version 3.3.2. Analysis of variance (ANOVA) tests were used to test for main and interactive effects of variety and damage on leaf Si concentration and normalised gene expression differences, ANOVA tests were also carried out to test for within variety differences between undamaged and damaged plants using the subset function. Linear models were used to test for assumptions of ANOVA (normality and homogeneity of variances) according to Crawley (2007). Silicon (%) values were arcsine transformed to meet assumptions of ANOVA. Significance was set to $P < 0.05$ for all tests. *Post hoc* Tukey tests were used to determine within treatment difference, significance was set to $P < 0.05$. Pearson's coefficient correlations were carried out to test for relationships between leaf Si (%) and stem and root dry weight (DW), leaf DW was not correlated as biomass was removed as part of the treatment. Packages used were lsmeans (Lenth, 2016), multcompView (Graves et al., 2015), ggplot2 (Wickham, 2009).

5.3 Results

5.3.1 Biomass and Si

Damaged plants had a higher leaf Si concentration than undamaged plants ($F_{1, 64} = 6.86$, $P = 0.04$; Figure 5.1 A). No differences in leaf Si concentrations were found between varieties ($F_{7, 64} = 0.68$, $P = 0.69$; Figure 5.2). Differences within individual varieties in terms of response to damage were also tested; however, no individual varieties responded to damage by significantly increasing their leaf Si concentrations. Overall the harsh varieties (when grouped together) had more Si deposited in the leaves compared to the soft varieties ($F_{1, 76} = 3.91$, $P = 0.05$). Silicon uptake ability (mg Si g^{-1} root DW) was also greater in damaged plants compared to undamaged plants ($F_{1, 63} = 9.04$, $P = 0.004$; Figure 5.1 B) and the N variety increased its Si uptake ability in damaged plants compared to undamaged plants ($F_{1, 8} = 5.16$, $P = 0.05$). No differences were found in Si uptake ability between varieties ($F_{7, 63} = 1.59$, $P = 0.15$).

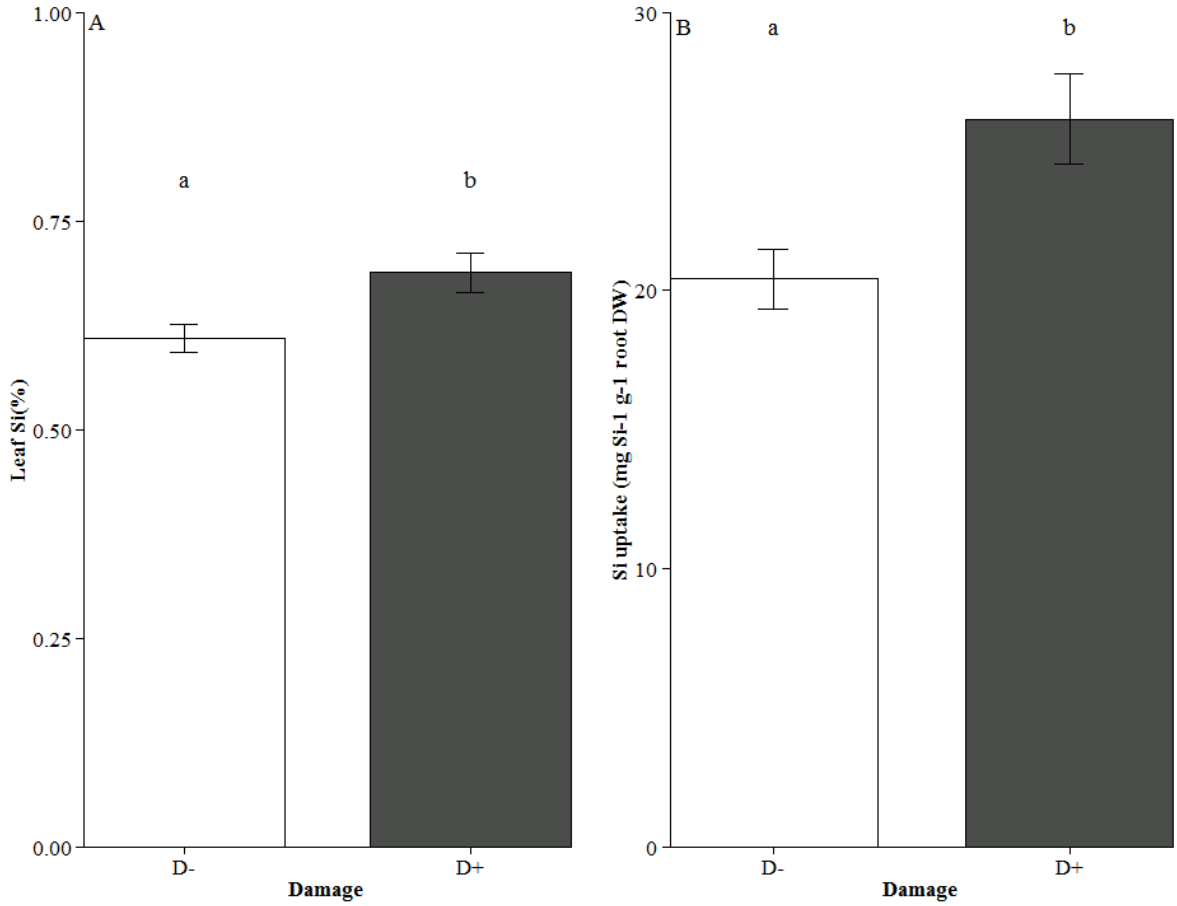

Figure 5.1: (A) Leaf Si concentration in undamaged (D-) and damaged (D+) plants of tall fescue. (B) Si uptake ability in undamaged (D-) and damaged (D+) plants. N = 79. Bars are mean values ± SE. Different letters denote significant differences between treatments (*post hoc* Tukey $P < 0.05$).

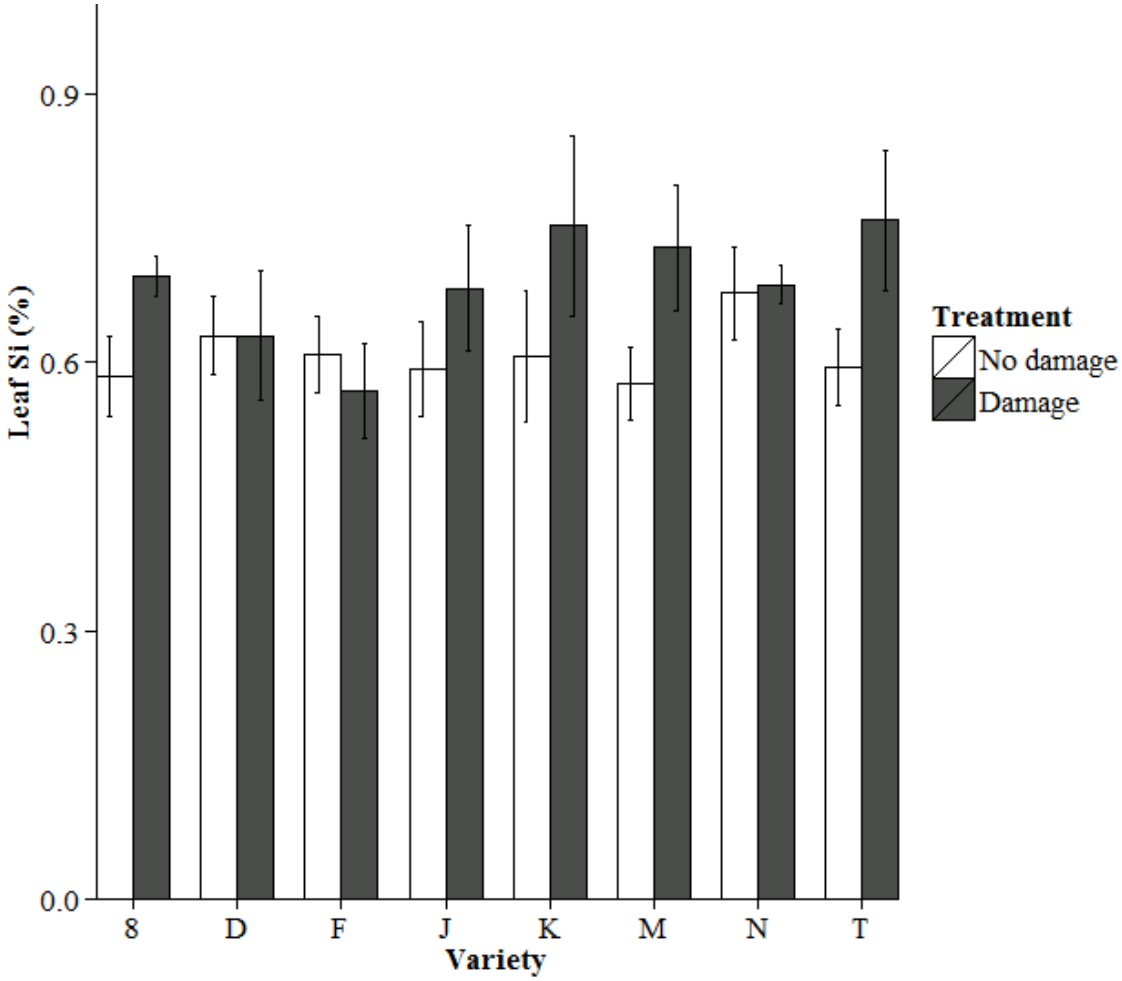

Figure 5.2: Leaf Si concentration in undamaged and damaged plants in 8 varieties of tall fescue. 8, D, F and J = soft varieties; K, M, N and T = harsh varieties. N = 79. Bars are mean values ±SE. No significant differences were detected in variety*damage interaction. No significant differences were detected between undamaged and damaged plants within each variety.

Undamaged plants had significantly greater root biomass ($F_{1, 64}$ = 21.79, P < 0.001) and stem biomass ($F_{1, 64}$ = 45.71, P < 0.001) compared to damaged plants. Negative correlations were found between leaf Si concentration and root biomass (t_{78} = -4.13, r = -0.42, P <0.001; Figure 5.3 A) and stem biomass (t_{78} = -6.33, r = -0.58, P < 0.001; Figure 5.3 B).

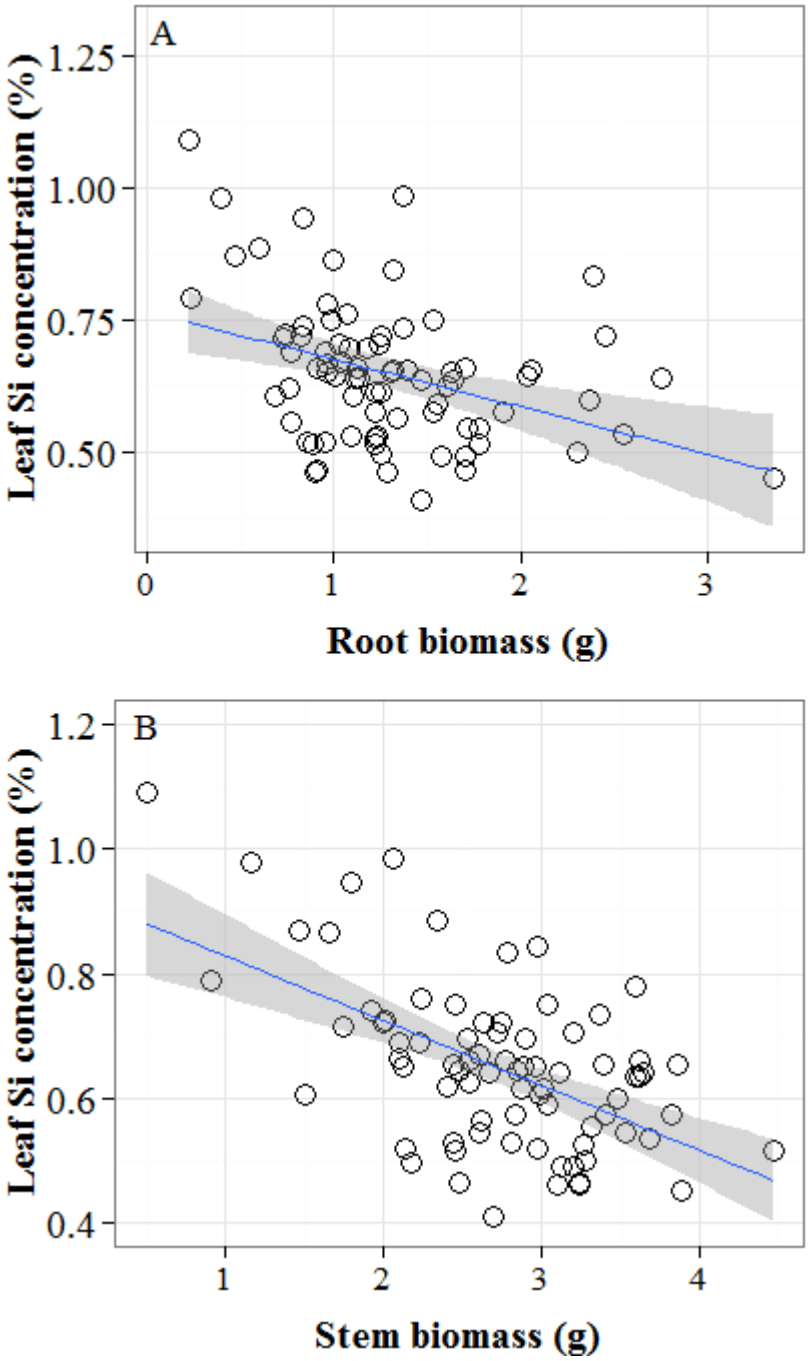

Figure 5.3: (A) Correlation between leaf Si content (%) and root biomass (g) and (B) Correlation between leaf Si content (%) and stem biomass (g). Solid line represents linear regression through the points. Shaded area represents 95% confidence intervals.

5.3.2 Silicon transporters and differential expression

Of the five Lsi1 and eight Lsi2 sequences that were designed RT-qPCR primers for, successful amplification and confirmation of amplification was carried out for one sequence for Lsi1 and one sequence for Lsi2 (Appendix 1, Table 5.2) herein referred to as tall fescue Lsi1 and tall

fescue Lsi2. The tall fescue sequence annotated as Lsi1 showed high sequence similarity to ryegrass (*Lolium perenne*) Lsi1 transporter (accession KY315994), BLAST results show 97% sequence identity and an E value = 0.0 and to barley (*Hordeum vulgar*) Lsi1 (accession AB447482), BLAST results show 89% sequence identity and an E value = 1e-97 (full transcript sequences for tall fescue Lsi1 are found in Appendix 1). Tall fescue Lsi1 also had 2 NPA motifs (Figure 5.4) with the precise 108 amino acid spacing between the NPA motifs (between the two regions highlighted in yellow, Figure 5.4), the ar/R selectivity filter (G-S-G-R – indicated by red boxes, Figure 5.4) and 6 predicted transmembrane domains (TMDs) which matched to both ryegrass and barley TMDs (Figure 5.5 A, B and C).

```
Barley          MASNSRSNSRATFSSEIHDIGTVQNSTTPSMVYYTERSIADYFPPHLLKKVVSEVVSTFL
Ryegrass        MSTNSRSNSRANFSNEIHDIGAARST--TPSMYYNERSIADYFPPHLLKKMVSEVVSTFL
Tall_fescue     MSTNSRSNSRANFSNEIHDISAARST--TPSMYYNERSIADYFPPHLLKKMVSEVVSTFL
                *::********.**.*****.:.:.:     :**.***************:*********

Barley          LVFVTCGAAAISAHDVTRISQLGQSVAGGLIVVVMIYAVGHISGAHMNPAVTLAFAIFRH
Ryegrass        LVFMTCGAAAISASDPTRISQLGQSVAGGLIVTVMIYSVGHISGAHMNPAVTLSFAVFRH
Tall_fescue     LVFMTCGAAAISSSDPTRISQLGQSVAGGLIVTVMIYSVGHISGAHMNPAVTLSFAVFRH
                ***:********: * ****************.****:***************:**:***

Barley          FPWIQVPFYWAAQFTGAICASFVLKAVLHPITVIGTTEPVGPHWHALVIEVVVTFNMMFV
Ryegrass        FPWIQVPFYWASQFTGAICASFVLKAVLHPITEIGTTVPHGPHWHSLVIEVVVTFNMMFV
Tall_fescue     FPWIQVPFYWASQFTGAICASFVLKAVLHPITTIGTTVPYGPHWHALVIEVVVTFNMMFV
                ***********:********************* **** * *****:**************

Barley          TLAVATDTRAVGELAGLAVGSSVCITSIFAGAVSGGSMNPARTLGPALASNRYPGLWLYF
Ryegrass        TLAVATDSRAVGELAGLAVGSSVCITSIFAGAVSGGSMNPARTLGPALASNHFTGLWIYF
Tall_fescue     TLAVATDTRAVGELAGLAVGSSVCITSIFAGAVSGGSMNPARTLGPALASNHFTGLWIYF
                *******:*********************************************:: ***:**

Barley          LGPVLGTLSGAWTYTYIRFEDPPKDAPQKLSSFKLRRLQSQSVAADD--DELDHIPV
Ryegrass        LGPVLGTLSGAWTYTFIRFEDPPKDAPQKLSSFKLRRLQSQSVAATDLEDDLEHIPI
Tall_fescue     LGPVLGTLSGAWTYTFIRFEDPPKDAPQKLSSFKLRRLQSQSVAATDLDDELEHIPI
                ***************:****************************** *  *:*:***:
```

Figure 5.4: Protein sequence alignment of predicted tall fescue Lsi1 with barley and ryegrass Lsi1 (top BLAST hits). Highlighted in yellow = NPA motifs. Red boxes indicate G-S-G-R selectivity Ar/R selectivity filter.

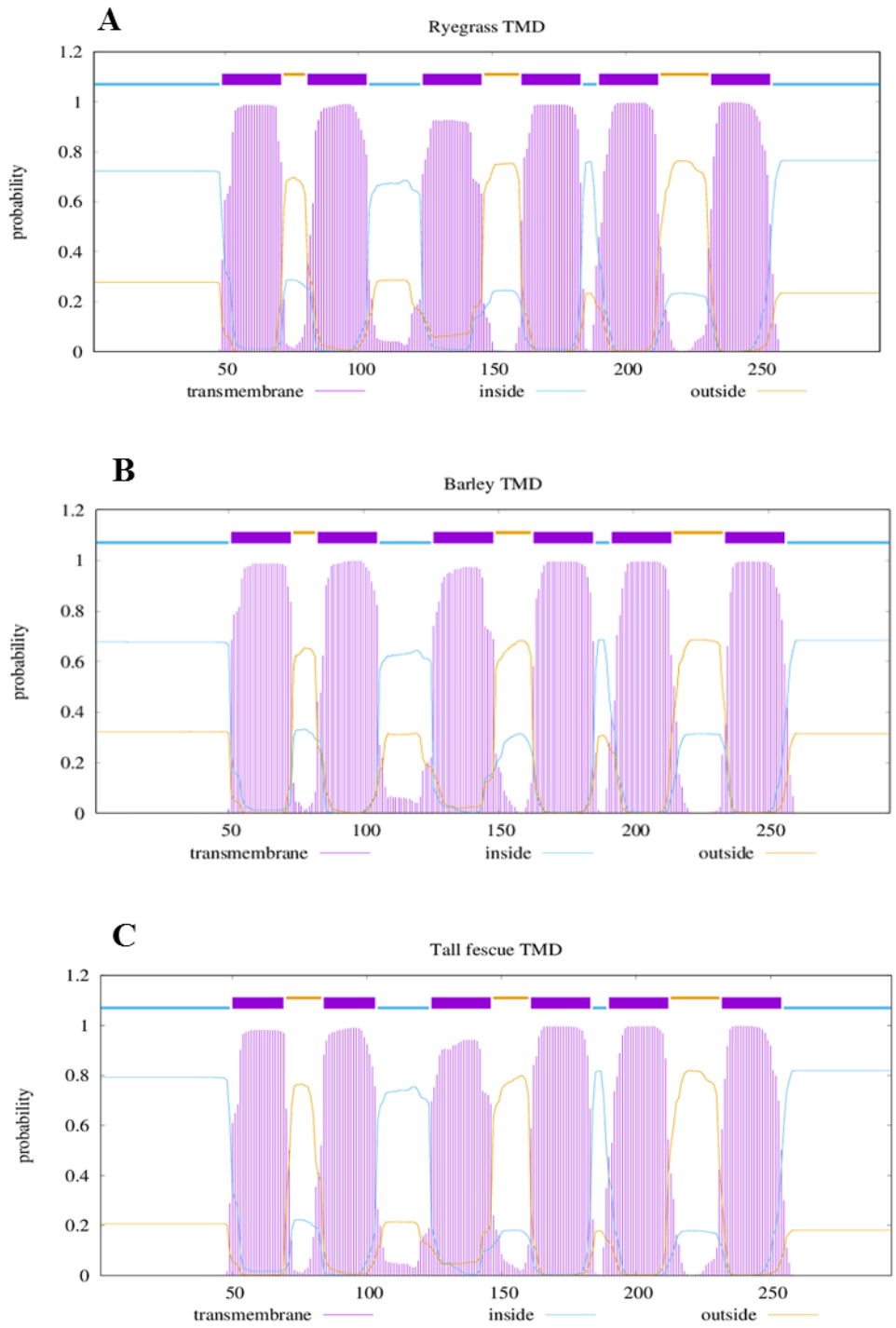

Figure 5.5: Distribution of the six predicted transmembrane domains (TMD) in Lsi1 sequences predicted by TMHMM for (A) ryegrass (B) barley and (C) tall fescue.

The annotated tall fescue Lsi2 sequence showed high sequence similarity to the barley (*Hordeum vulgare* L.) Lsi2 transporter (accession BAH84976.1), BLAST results show 94% sequence identity and E value =0.0. The tall fescue Lsi2 sequence had 11 predicted TMDs, typical of the Lsi2 Si transporters, and was similar to the barley transmembrane domains, which also had a prediction of 11 TMDs (Figure 5.6 A and C). The predicted protein structures of barley Lsi2 and tall fescue were overall very similar (Figure 5.6 B and D). The conserved

domains of the annotated tall fescue Lsi2 also aligned to accession PLN00136 (a provisional Si transporter in rice).

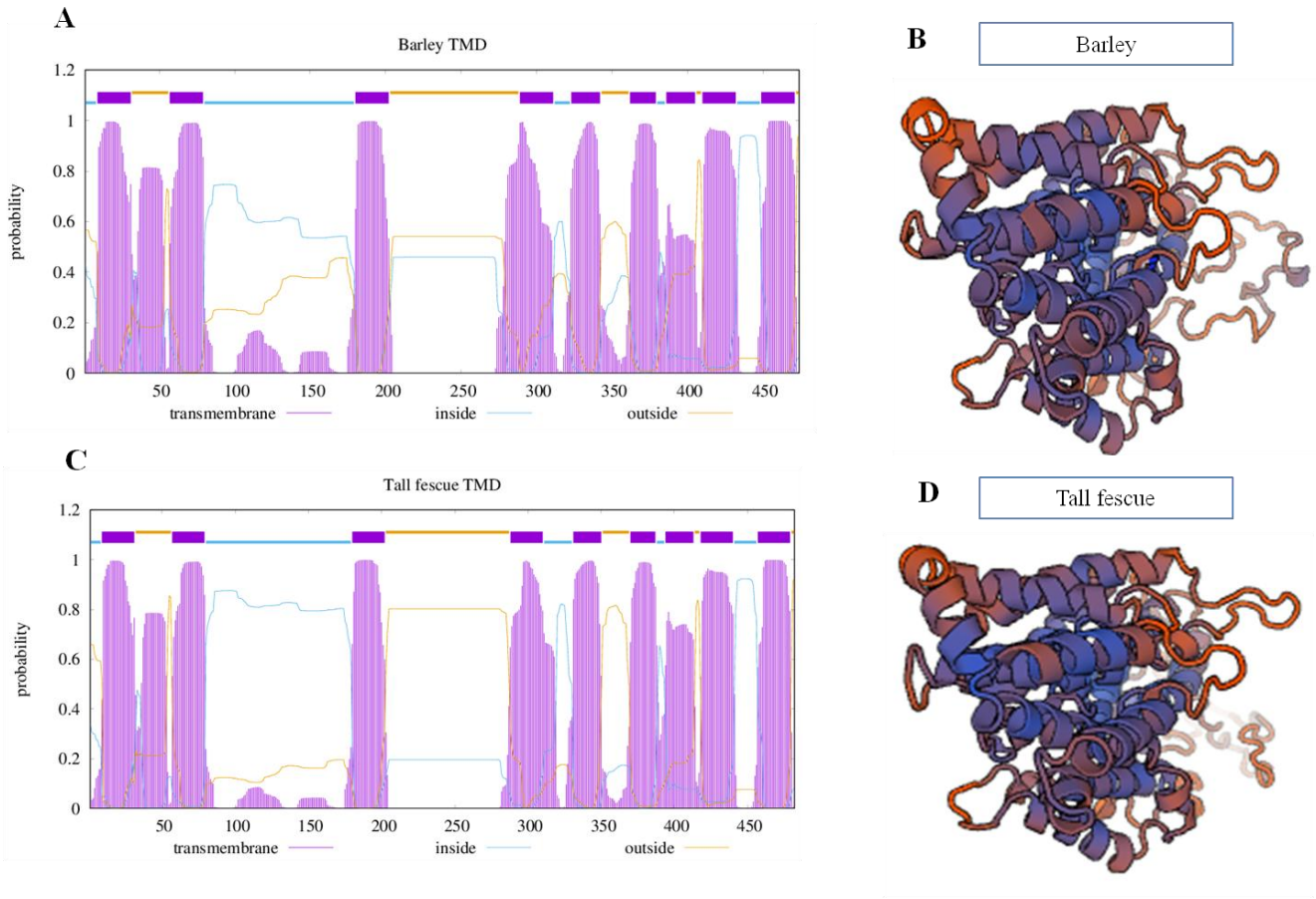

Figure 5.6: Distribution of the eleven predicted transmembrane domains (TMD) in Lsi2 predicted by TMHMM for (A) barley and (C) tall fescue. Three dimensional structure of Lsi2 protein in (B) barley and (D) tall fescue

Comparison of the expression of tall fescue Lsi1sequence showed no significant changes in expression between varieties ($F_{7, 32} = 0.74$, $P = 0.64$) or between undamaged and damaged plants ($F_{1, 32} = 0.65$, $P = 0.43$). Comparison of the expression of tall fescue Lsi2 sequence between varieties in undamaged and damaged plants showed no main effects (variety: $F_{7, 32} = 0.52$, $P = 0.81$ and damage: $F_{1, 32} = 0.79$, $P = 0.38$), but a marginally non-significant variety*damage interaction was found in Lsi2 expression ($F_{7, 32} = 2.28$, $P = 0.05$; Figure 5.7) though differences between treatments were not detected using *post hoc* Tukey. No correlations between the

relative expression of tall fescue Lsi1 or Lsi2 sequences and leaf Si concentration were reported (t_{46} = -0.47, r = -0.07, P = 0.64 and t_{46} = -0.53, r= -0.08, P = 0.60).

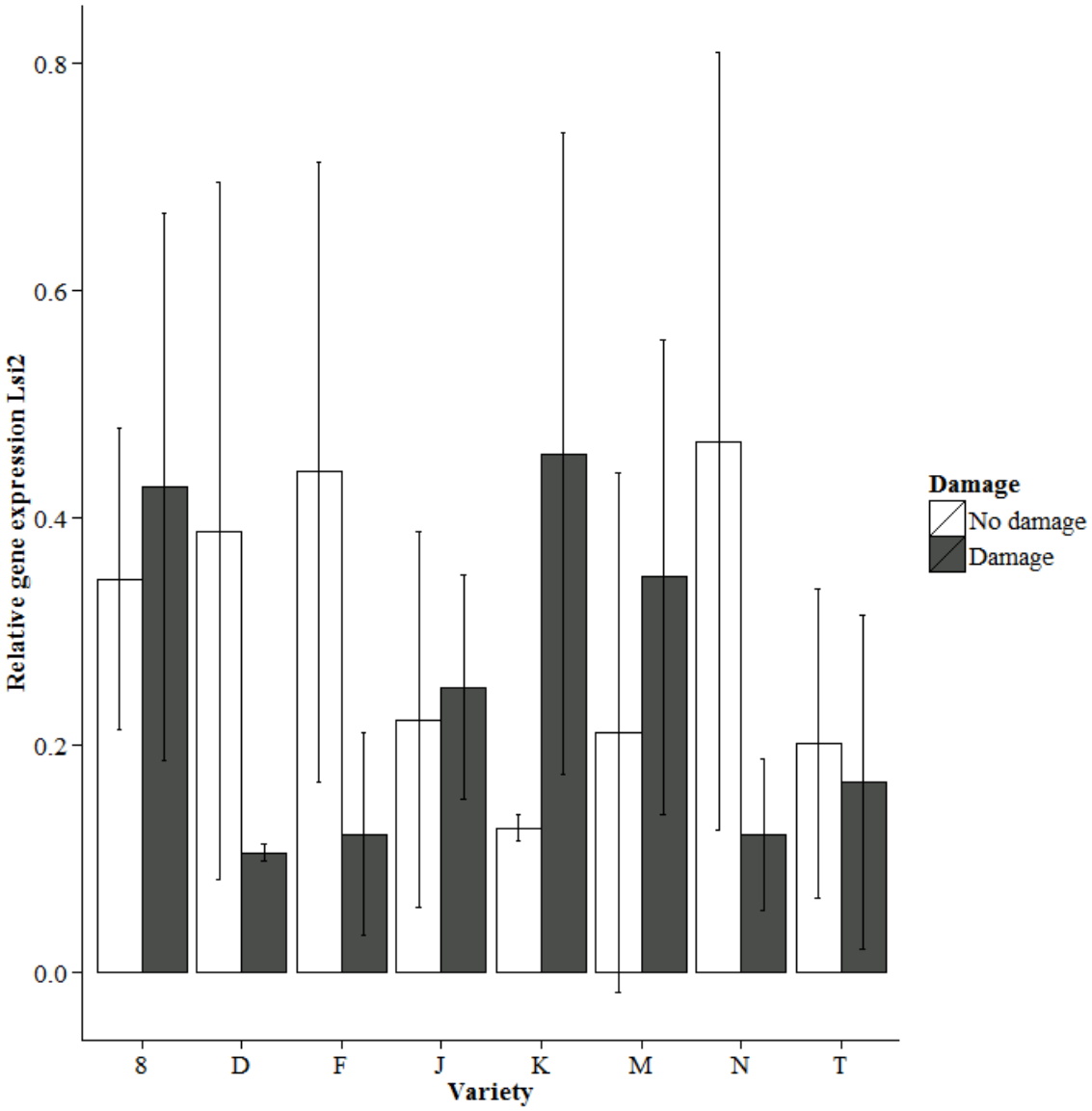

Figure 5.7: Relative gene expression of tall fescue *Lsi2* in undamaged and damaged plants. 8, D, F, J = soft varieties; K, M, N, T = harsh varieties. No difference were detected between undamaged and damaged plants. Bars are mean values ± standard deviation.

5.4 Discussion

In contrast to previous results (Hartley et al., 2015; McLarnon et al., 2017), this study did not find any difference in leaf Si concentrations between varieties. There was an increase in Si accumulation into the leaves when plants were damaged compared with the undamaged plants. It was also found that damaged plants had improved Si uptake ability compared to undamaged plants (i.e. they were able to take up more Si per gram of root). Undamaged plants had greater root and stem biomass (DW) compared to damaged plants, and leaf Si concentrations were negatively correlated with these biomass fractions, suggesting a trade-off between growth (in terms of biomass) and Si accumulation. The active Si transporter (Lsi2) has been demonstrated to be driven by a proton gradient in the plasma membrane, actively transporting Si in the root cells (Ma et al., 2007a) and this potential trade-off, between biomass and Si uptake, has been observed previously (Chapter 3; Simpson et al., 2017), and the negative relationship between growth and Si concentration in damaged plants, suggests this trade-off is linked with the metabolic cost associated with Si uptake from Lsi2. The two tall fescue Si transporter sequences (Lsi1 and Lsi2) were aligned to barley Lsi1 and Lsi2 and appear to have similar properties to previously characterised Si influx and efflux transporters. Silicon concentration in the leaf did not correlate with expression of either tall fescue *Lsi1* or *Lsi2* in the root. Previous studies where a positive relationship between Si and relative transporter expression was reported found this relationship within 24 hours, they also found that the relative expression of *Lsi2* decreased when Si was constantly supplied after seven days (Mitani et al., 2009b); this study lasted 19 weeks, therefore it may not be surprising no correlation between leaf Si concentration and root Si transporters was found, as increased Si transporter expression may only be a temporary reaction to changes in Si availability.

5.4.1 Induced Si-based defenses in the leaves of damaged plants

It was hypothesised that damaged plants would take up more Si than undamaged plants, and that varieties described as harsh would take up more Si compared to soft varieties. This study did not find any differences between the varieties in terms of the leaf Si concentrations. These results are surprising given that previous work (Hartley et al., 2015; Chapter 3; McLarnon et al., 2017) has found varietal differences. Liang et al. (2006) found that species exhibit both active and passive Si uptake, depending on external Si concentrations. Under constitutive Si supply, *Lsi1* and *Lsi2* in maize and barley were down-regulated after seven days (Mitani et al., 2009a, b). Although Si was added to the growth medium, this was also the case in McLarnon et al. (2017) where there was a difference in Si transporter expression, so it is unlikely that Si supply the main reason for these differences, instead it may be due to the role the potential isoforms of Lsi1 and Lsi2 tested in this study. These sequences were different to those tested in McLarnon

et al. (2017) and it may be that the isoforms tested here have a smaller role in Si uptake than those previously found to be differentially expressed.

When the varieties were separated into harsh and soft groups, there was a marginal difference between the two groups –the harsh varieties accumulated more Si, suggesting that although Si uptake and deposition has some degree of plasticity between the varieties, the harsh varieties are able to accumulate more Si than the soft varieties. Genotypic differences in Si uptake and accumulation have been reported in other studies (Wu et al., 2006; Ma et al., 2007b; Hartley et al., 2015, McLarnon et al., 2017). Similar results were found in this study compared to previous work (chapters 3 and 4): damaged plants had significantly higher leaf Si concentrations compared to undamaged plants. Surprisingly, no differences within the varieties between undamaged and damaged plants were found, but overall there was a difference between undamaged and damaged plants. These results suggest that Si uptake in this species is variable and, although no significant differences were observed in varieties between undamaged and damaged plants there was a trend for higher Si concentration in the leaves of harsh plants compared to the soft plants.

5.4.2 Silicon transporters and transporter expression

It was also hypothesised that damaged plants would express the Si transporters at a higher level to the undamaged plants, and that harsh varieties would have higher expression levels of the Si transporters compared to the undamaged plants. BLAST results from the tall fescue Lsi1 and Lsi2 (Chapter 4) sequences show high similarity to fully sequenced genes (barley: Mitani et al., 2009b and Chiba et al., 2009 and ryegrass: Pontigo et al., 2017). Sequencing of the PCR products confirmed the primers would amplify the regions of potential *Lsi1* and *Lsi2* genes in tall fescue.

No differences between the expression of *Lsi1* and *Lsi2* between varieties or between undamaged and damaged plants were reported here. There was a marginally non-significant interaction between variety*damage in *Lsi2* expression (Figure 5.7, $P = 0.05$), though *post hoc* tests did not detect any differences in the interaction. The large variation in expression of *Lsi2* was likely due to the nature of the biological replicates used: each sample represented one biological replicate only and there were large differences between these replicates in terms of expression, reflected in the results (Figure 5.7). The large variation may be due to the polyploid nature of *F arundinacea*, where individual plants may differ in the number of copies of the same gene, thus further study using technical replicates as well as more biological replicates would be useful to remove these differences between individual plants. These results are surprising, given that differences were detected between two varieties of tall fescue in terms of *Lsi2* expression in

a previous study (RNA-Seq data: McLarnon et al., 2017). However, attempts to successfully design primers for all of the potential *Lsi2* isoforms that were found to be upregulated in McLarnon et al. (2017) failed. Given the complex molecular structure in tall fescue, an outbreeding allohexaploid (Gibson and Newman, 2001; Hand et al., 2012b), that potential isoforms of the same gene were found is not surprising, due to the potential for single nucleotide polymorphisms and splice variants. Further, as this study did not involve proteomics, it is not possible to comment on the function of these proteins in inducible Si-based defenses; low transcript expression does not necessarily reflect the protein function. Future work into the protein structure of these potential genes needs is recommended in order to understand how the protein is expressed in these conditions.

The potential tall fescue *Lsi2* isoform analysed in this study was not differentially expressed in previous work (though it was highly expressed between the varieties), and so more work is needed on the protein activity of this sequence. Root sample location has been found to be an important factor in influencing expression of both *Lsi1* and *Lsi2* (Soukup et al., 2017; Yamaji et al., 2008). Samples were taken from the top 5cm from where shoot meets the root, and it may be that Si transporters are not highly expressed in this region. Previous work on sorghum has found that this particular region has low Si transporter expression when compared to the root apex (Soukup et al., 2017). However, this contrasts to studies in rice, where *Lsi1* expression correlated with increasing distance from the root tip (Yamaji et al., 2008). Previous work (McLarnon et al., 2017) also sampled from the same region as this study, but found that the Si transporters were differentially expressed. This suggests that Si transporters are found in different densities and expressed differently within the root (spatially), and this appears to vary between species.

It was expected that the Si transporter expression would be higher in damaged plants as inducible Si-based defenses would become active and stimulate an increase in expression to explain the increased leaf Si concentration. These results suggest that in the presence of constitutive Si supply, passive means of transport may be favoured over active means, though expression of the active Si transporter was still found. Soil-available Si concentration clearly affects Si transporter expression (Yamaji et al., 2008; Vulavala et al., 2016) and it seems this may be why there is such high variation between and within species. Other studies have reported that expression of Si transporters are either upregulated (Ye et al., 2013; Kim et al., 2014; Pontigo et al., 2017), downregulated (Yamaji and Ma, 2011), or both (Vulavala et al., 2016) depending on the Si transporter, plant species, Si concentration and stress applied.

However, damage induced increases in Si-based defenses appear to be more complex than simply increased expression of the transporters involved in Si uptake, and to date few studies have looked into the molecular components of inducible Si-based defenses from the perspective of leaf damage. It is known that this species does upregulate Si transporters, though these

differences were found between varieties (McLarnon et al., 2017). This study did not measure transporter density – this has previously been found to be the causal difference between rice genotypes in terms of constitutive Si transporter expression (i.e. expression of transporters in the absence of abiotic or biotic stress) (Ma et al., 2007b) – it may be that the density of the active transporter tested in this study is found in low abundance in the root and may explain why expression values for it were very low. Previous studies have found that time is an important factor in the expression of *Lsi2* expression levels (Yamaji and Ma, 2011): *Lsi2* expression was the highest at 11 weeks and the lowest at 14 weeks. This study lasted for 19 weeks, which may suggest why we saw such small differences in transporter expression. However, Ye et al. (2013) found that expression of both root transporters were upregulated in response to herbivory and we expected a similar result. This study did not use actual herbivores, and previous evidence has suggested that herbivores may secrete olfactory cues in their saliva which plants use as a signal for inducing plant defense (Arimura et al., 2005). Transcript levels do not reflect protein activity and therefore, further work into the proteomics of these genes is necessary to fully understand their role in inducible Si-based defenses.

5.5 Conclusions

This study investigated the effects of damage of eight varieties of tall fescue to understand the molecular basis of induced Si-based defenses in this species. Damaging plants increased their leaf Si concentrations, but no differences in Si concentrations were observed between varieties, contrasting with previous findings and suggesting Si uptake in this species is variable. Negative correlations between leaf Si concentrations and both root and stem biomass (DW) were found in damaged plants, suggesting a cost associated with Si uptake. Silicon transporter expression did not differ between undamaged and damaged plants, and no differences between transporter expression between varieties were found. Tall fescue is an outbreeding hexaploid, so it is likely that more than one isoform of Si transporter is responsible for Si uptake in this species. It seems likely that Si transporter expression occurs quickly after damage events, therefore a time-course experiment is required to further investigate the expression of these transporters in induced Si-based defenses. It seems that Si accumulation happens in response to damage, but as for how long the transporters remain highly expressed still remains to be answered. The findings of this study suggest a temporal and localised expression of Si transporters exists in this species, but further investigation is necessary to full understand how this species accumulates Si following foliar damage.

5.6 Appendix 1

Table 5.2: Transcript sequences for potential Lsi1, Lsi2 and eEF1 transcripts. Highlighted regions indicated amplified regions for RT-qPCR.

Identifier	Transcript
TRINITY_DN45085_c2_g2_i1	TTTATTTAATAAGAGATTTGAATTTTGCAAGCAGGCACACAAGATAACATTACTAATTAACTTCCTTACATCTTGGCATTACAGATG CATGCAGCCACGTGCCTGCACATAAAACACAGTATATGTATGTGCGTCCTTTGGAGCAAATTAGCTAACCCATGCTGACCAATCACC TCGGTGCACTGCTTAATTAATTAGCACACACCGTGGTATATATGGATCTTCATCCCTTGATTCTTCTCTAATCTTCGGTCGGTCGTTA CATACGTACACAGCAACTTACACATACCAAACAGAAGCTAACACCTATACATACGTACAAGTGTCCATATATACTCTGTGTATGTAT GTACGTACATACGGGCAGGTGTAATATGAAAGTATTGCAACATATATAGTTGGATCGGCCACAGCGGGCGGCGGTGAGGTCTACAT CTTTCCGATGAGGGGGATGCCGACGGCGGTGACGATGAGGGTGGAGGGGACGCCGAAGATGAGGTGGTTCCAGAAAGTGAGCTCG TAGGCGTTGCGCGGGGCACGGCGCGCCTGCTCGCACACGATCAGGTTCGCCGCCGATCCTAGCAGCGACAGGTTCCCTGCCACTGT GCTCACCCACGCCAGCAGCAGCCACGACCGGGTCACTGCCGCCGGGGAGATGAGCGCCGCCGCGGTTGCCACCTCGTTCCCCATCA GCAGCACTGTTGGTACGTTGGAGGCGAGGTTGGAGAGGAGGAGGATGATGATGGAGAGGACGGAGATGCCGCCGGCGCTGTCGAC CTTGGAGTAGGGCGCCATGAAGTTCCAGATCGCGCTGGGCAGCCCCGTCTTGTTGAACCCGCTTACCGTGATGAACATCCCGGAAA AGAAGACGAGCAGCGAGTACGACACCTTCACCAGGCAAGGCTCAGCGTCCCGGAAGTCGACAACGACAAGCGCGATGGCCGTGGT GATGGCCGTCCACGACATGTTGAGCCCGACCATGTATGCGACCACCATGCCCACGGTGACGATGTAGGCGAAGCTCTTGAGGAAGA GCTTCCGGTGGTGCTCCGTGCACTGCATGAACCACGGGTGCTTCGTCGAGAGGTTCTCCTCCATCATCGACTCTCCGTCGTCGTTGAT GGTGCTGCCGTTGAGAGGGTTGAGAGTGTGCGGGCTCTTGAGCGGGCTCTTGAGGGACGCGATTGAGGGTGACCGGCCTTCCTCGG CGGCGTGCATCTGCTTGCCGGCGTCAACAGCGTCGGGGGAGACGCCCTCGAGGTCCTTCCAGTACATGCAGAGCAGCATGACCATG TTGACGGCCATGCCGGCGAGCATGGCCGGCAGGATACCCAGGAGGAACCTCGGGAAGGAGATCTTGCTGTTGAAGGCGATGACGA GGTTCTGCGGGTTGCCGATGGGGGTGGCGCTGGACCCGATGTTGGCGCTGGAGGCGAGGGCCAGGAGGAAGGGCTTGGCCGGGAG GTTGCGCTCGGCGGCGAGCTCGAGGACGAACTCGGTGAGCACGACGCAGCAGGTGTCGTTGGTGAAGAGCGCCGAGGCGAGGGCC GTGACGACGCAGACGCGGCAGAGGAGGTCGCGGCCGCCCTGGCTCTTCCAGGCCAGGAGGGTGCCGAGGTGCTTGAACATGCCGG CGTTCTTGAGGTAGCCGCCCACGACCATGGTGGAGAAGAGGAGGCCGAGGATGGGGAGATCGATGGAGGCGTAGGCGTCGTCCGG GGATATGACGTGGAAGACTATCATGAGGACCGCCGAGAGGAGGGACCCCGCAGTGCGGCCGATGGGCAGGAACGGTACGGACGG GAACACCGCCATCATCCAGAACACGGCGAAGGCCACCGAGCCGAGGATCACCTTGGGGAGAGACGCGAGCGCCATGGCCGATCGT TGGATCGAGCTCCTGGTAATCTCTAGTTAGAGCTTTGGTTAGTGAGAGTGTGAGACACCTAGGTCGATCGGCTGCTGGGTAGTGAGT GAGCTTCGTGCTGCCGATGGAGGAGGTCGTGGACGAGGGTATAAGAAGAGCTTGGCTGGGTGGCTAGCGAGGTGTGTATGATGCC ACGTAGGCCGGCCGGGTTGCTTACTTTGGTTACTCGAGCCTAAGAGACATAG

TRINITY_DN41992_c1_g2_i1	CTTTTTTTTTTGTTTTCTCCAAGAACGAAAGCAGGGATGTATTATGTACTGCAACTGCAGGAGAGATACACTTGGCACATGTGCACG CACACATACGCCGCTTGCGATCGACTGCAGAGACCCAGCCAAAATCTGAACTTCGCACAGGCCAACAGAGCGAGAGCAATCTACA GCTAACACATGTGCGCACGCACGCGCGTACTCCCCGGCTCATCGGCACAAGTGACACACACACAGGACACACGCACACACATGTCG CTAGCGACGACGACGCAACGGCGATCAGATGGGGATGTGTTCGAGCTCGTCGTCAAGGTCGGTTGCGGCGACGGACTGGCTCTGCA GCCGGCGGAGCTTGAAGGAGGAGAGCTTCTGCGGCGCGTCCTTGGGCGGGTCCTCGAAGCGGATGAAGGTGTAGGTCCAGGCGCC GGAGAGCGTGCCGAGGACGGGGCCGAGGAAGTAGATCCAGAGGCCGGTGAAGTGGTTGCTGGCCAGCGCCGGGCCCAGCGTCCTC GCCGGGTTCATCGATCCACCCGACACCGCCCCTGCGAAAATGGACGTAATGCAAACAGAAGATCCGACAGCTAACCCGGCCAACTC ACCCACCGCTCTGGTGTCCGTTGCGACGGCGAGCGTGACGAACATCATGTTGAAGGTGACGACGACCTCGATGACGAGCGCGTGCC AGTGCGGGCCGTACGGCACGGTGGTGCCGATGGTGGTGATGGGGTGGAGCACCGCCTTGAGCACGAATGACGCGCAGATGGCGCC CGTGAACTGCGACGCCCAGTAGAACGGGACCTGAATCCATGGGAAATGCCGGAAGACGGCGAAGGAGAGGGTGACTGCAGGGTTC ATGTGCGCGCCGGAGATGTGTCCGACGGAGTAGATCATCACGGTCACGATGAGCCCGCCGGCGACCGACTGTCCCAGCTGCGATAT GCGTGTGGGGTCGCTGGAGCTGATGGCCGCCGCTCCGCAGGTCATGAACACCAGCAGGAACGTCGACACCACCTCCGACACCATCT TCTTCAGGAGGTGGGGCGGGAAGTAGTCCGCGATAGACCTCTCGTTGTAGTACATGCTGGGCGTGGTAGAGCGCGCCGCGCTGATG TCGTGGATCTCGTTCGAGAAGTTCGCCCTGGAGTTCGATCTCGAGTTGGTCGACATTTCCGACGACGAACTTGCACACGAAGGAGG AGGAGGGGGAGGAGGAGGAGAAGCTCTCTTGTGGTCTAG
TRINITY_DN28909_c0_g1_i1	GTGCTCACGGGTCTGGCCATCCTTGGAGATACCAGCCTCAAAACCACCAGTGGTGGAGTCAATGATGAGCACAGCACAATCAGCCT GGGAGGTACCAGTAATCATGTTCTTGATGAAGTCACGGTGTCCAGGGGCATCAATGACTGTGCAGTAGTACTTGGTTGTCTCGAATT TCCAGAGGGCAATATCAATGGTGATACCCCTCTCACGCTCAGCCTTGAGCTTGTCAAGCACCCACGCGTACTTGAATGACCTCTTGT TCATCTCAGCAGCTTCCTTCTCAAACCTCTCGAT

6 Chapter 6: General Discussion

6.1 Summary of aims

Festuca arundinacea is a cultivated forage grass known for its unpalatability and rough leaf surfaces (Gibson and Newman, 2001; Lou et al., 2015). Reports of the Si concentration in this species in the literature show that it does not accumulate particularly high quantities of Si in its leaf tissue (mean value =1.31%; Hodson et al., 2005). Despite this, it remains unpalatable, such that animals offered mixed swards avoid it (Gibson and Newman, 2001). Therefore, understanding the role of Si in different leaf textures, in terms of concentration and deposition of Si is necessary for selection of traits in plant breeding programmes developing new varieties of forage grass. The main aim of this work was to understand the relationship between Si and leaf texture in a number of breeding varieties in tall fescue, so improving understanding of traits that could be used for targeted selection. A range of varieties described over a scale of "very very soft" up to "harsh" were provided for study. Silicon transporters have been characterised in many crop species such as barley, maize and wheat (Chiba et al., 2009; Mitani et al., 2009a, b; Monpetit et al., 2012), yet Si transporters have not been characterised in tall fescue. As well as determining if Si transporters exist in tall fescue, the mechanisms behind inducible Si-based defenses within this species were investigated, with a particular focus on how these related to leaf texture, and therefore genotype (Figure 6.1).

6.2 Silicon influences leaf texture in tall fescue

It was hypothesised that harshness would be related to Si uptake and deposition: varieties described as harsh were hypothesised to accumulate more Si and deposit it in abrasive forms such as within trichomes. This deposition would contribute to the harshness associated with the leaf texture. The results were more complex than was hypothesised: the harsh varieties did have more Si in the leaves than the soft varieties, though this was not always the case (see chapters 4 and 5). Furthermore, imaging of the Si deposition highlighted an important finding: the leaf texture was influenced by the types of structures that were silicified, rather than quantity of Si alone. The harsh varieties generally silicified more structures, and these structures appeared to be contributing to leaf texture (Hartley et al., 2015). Studies on how Si influences leaf texture in this species (and indeed other species) are limited, but Cougnon et al. (2016) found that there was no relationship between Si content and softness, which is in agreement with these findings

(Hartley et al., 2015; chapter 3), since it was reported that silicification of trichomes appears to be one of the main drivers of leaf texture in this species, rather than Si content itself.

Tall fescue deposits Si into rounded phytoliths in the form of silica short-cells, characteristic of the *Festuca* genus (Piperno, 2006) and these may be less effective at causing the abrasive leaf texture than the trichomes (Hartley et al., 2015). This thesis found that trichome density was influenced by Si supply, and this has been previously reported by Meunier et al. (2017), who suggest that trichomes act as a reservoir for excess Si. However, the results of chapter 3conflict with this idea, because chapter 3 found a negative trend for phytolith deposition when Si concentrations were higher, and a positive correlation between trichome density and Si concentrations which suggests that at lower Si concentrations, phytoliths may be a "cheaper" alternative to trichomes. This evidence suggests trade-offs within Si-based structures exist: varieties that take up more Si invest more in trichomes than the softer varieties.

Further, Hartley et al. (2015) found that structural deposition was more important for surface texture and abrasiveness than overall quantity of Si accumulated: *F. ruba* had the highest leaf Si concentration of all four grass species tested, yet it silicified fewer structures compared to the other species. Trichomes slow down food intake by forcing insects to chew around them (Hanley et al., 2007; Strömberg, 2016) and therefore may be one of the mechanisms causing insect herbivores to actively avoid tissues high in Si. Increased processing time of plants high in Si may explain why grass palatability to sheep is related to Si content, with higher Si content reducing the bite-rate in sheep foraging on Si-supplemented grasses subjected (Massey et al., 2009). In insects, high Si levels have been shown to abrade insect mandibles, so reducing digestive efficiency and growth rate (Massey and Hartley, 2009), whilst Reynolds et al. (2016) hypothesised that ground wollastonite ($CaSiO_3$) did not have a direct impact on larval insect growth, despite contributing up to 3.3% Si, due to the potential removal of the abrasive properties of Si in the grinding process of $CaSiO_3$.

The work from chapters 2 and 3 provide novel understanding in terms of how Si contributes to leaf texture, and has demonstrated, for the first time, trade-offs between different types of Si-based structures. However, the mechanisms by which Si influences trichome production in this species remain unclear: chapter 3 showed that when additional Si was supplied, more trichomes were produced, yet no correlation between foliar Si concentration and trichome density was found. Further work, for example a molecular study of the genes involved in trichome production, is needed to fully understand the relationship between Si supply, trichome production and plant defense against herbivores. Whether Si is contributing to foliar palatability through trichome and phytolith production alone or through these mechanisms in combination with others remains unclear, particularly for larger herbivores such as sheep and cattle.

6.3 Trade-offs between Si and C-based leaf traits exist

It was hypothesised that trade-offs between Si and carbon (C) allocation would exist in tall fescue, as has been found in other species (for example Frew et al., 2016). As there is a cost involved in Si uptake by Lsi2, this cost could be offset by reducing C-based structures involved in the cell wall, and therefore a negative relationship between Si and C-based leaf structural traits would be found. The concentration of C was higher in the softer variety and Si was higher in the harsh variety (chapter 2), suggesting there is such a trade-off between Si and C. A negative relationship between lignin and Si was also found (chapter 3). Trade-offs between Si and C have been reported previously (Cooke and Leishman, 2011b; Cooke and Leishman, 2012; Schoelynck and Struyf, 2016; Frew et al., 2016; Ryalls et al., 2017) and it has been suggested that C-based structures are energetically expensive to make, and Si may be able to fulfil this structural role (Raven, 1983; Cooke and Leishman, 2011b). Silicon is taken up in greater amounts in short-lived leaves compared to long-lived leaves, which invest more in C-based structural support (Cooke and Leishman, 2011b). Frew et al. (2016) found that root phenolics were produced in smaller concentrations when higher amounts of Si was found in the plants.

The negative relationship between lignin and Si found in chapter 3, suggests that there may be some trade-offs between Si and C occurring within tall fescue. Correlations between lignin and Si are conflicting in the literature, where they are sometimes found and other times not, and reported as positive or negative, depending on the study (Cooke and Leishman, 2012; Schoelynck and Struyf, 2016) and it appears that this variation is not only found between species and environmental conditions, but also occurs between varieties of the same species (chapter 3). Such a relationship also suggests that Si may be used preferentially over lignin as it is a cheaper structural alternative (Raven, 1983) and given that more C in plants with less Si (chapter 2) was found, this may be true for tall fescue. However, when considered with the evidence found for the higher Si accumulating varieties having reduced biomass, the negative relationship between Si and lignin may be due to cost involved in Si uptake, which has been found in another recent study (Simpson et al., 2017) and in chapters 3 and 5. These findings here support the hypothesis that Si and C trade-offs exist in tall fescue; Si uptake comes at a cost to the synthesis of C-based structural components in high Si accumulating varieties (

Figure 3.8).

It was also found that ferulic acid (FA) was positively correlated with leaf Si concentration, these findings suggesting that Si may be adopting a structural role within cell walls by cross-linking with FA, which normally cross-links with lignin (Soukup et al., 2017). However, this is speculative as there is still no clear evidence that Si-C links form in nature (Guntzer et al., 2012), although the fact there was no correlation between lignin and Si in damaged plants, but

still a positive correlation between Si and FA does suggest that Si may be enhancing the structure of the cell wall in damaged plants.

Further work investigating the relationship between Si and leaf structural traits is necessary, in order to fully understand the structural role Si has within the cell wall, and if this role is linked with inducible Si-based defenses.

6.4 Molecular and physiological evidence for inducible Si-based defenses

It was hypothesised that plants that accumulated more Si in response to damage, would do so by actively increasing Si uptake, and this would be reflected in the higher gene expression of Si transporters in plants with higher leaf Si concentrations. Few studies have investigated whether increases in Si induced by damage are passive, i.e. achieved via increased transpiration rates, or active, i.e. involved additional energy-requiring processes, such that transpiration alone cannot explain the increases in Si post-damage. The results showed that transpiration has a role in the passive uptake of Si, and is partially responsible for the varietal differences in Si uptake found, but transpiration alone could not explain the differences in Si found between undamaged and damaged plants when the transpiration rates were equal. Many studies report similar findings in terms of evidence of active Si uptake: for example Si uptake has been found in the absence of transpiration (Kumar et al., 2016) and Si is reported to be found in areas of low transpiration, such as the husk (Yamaji et al., 2015).

Ye et al. (2013) found an increase in expression of *Lsi1, Lsi2* and *Lsi6* in response to herbivory, and this is the only study (to my knowledge) that has looked into Si transporter expression in relation to herbivory. The results of Chapter 5 showed that Si was taken up more in damaged plants than undamaged, but these differences were not reflected in the expression of the Si transporters, though an increase in expression of *Lsi2* was found in the harsh variety in damaged plants (McLarnon et al., 2017). The study by Ye et al. (2013) was carried out in the presence of herbivores and suggests that inducible Si-based defenses may be more honed into the signalling molecules known as herbivore-induced plant volatiles when herbivores chew on plant tissue as previously suggested (Kvedaras et al., 2010; Reynolds et al., 2016). Many studies have found evidence of increased Si in response to herbivory (for example Massey and Hartley, 2006; Massey et al., 2007a) but the mechanisms behind these increases remain unclear. The lack of change Si transporter expression in chapter 5 suggests that there may be specific splice variants of the Si transporters in this species, and given that it was not possible to test for differences in the same transporters that were differentially expressed in McLarnon et al. (2017) it seems likely, especially given the complex nature of the genome involved in tall fescue. Further,

transcript expression does not necessarily reflect protein expression. Clearly, Si is being accumulated in the damaged plants in higher quantities than the undamaged plants, but the mechanisms of this increased Si concentration remain to be found. It is likely that inducible Si-based defenses are controlled post-translationally, in light of these findings, and therefore proteomic work needs to be carried out in order to fully understand inducible Si-based defenses in this species, and for all of the isoforms isolated in McLarnon et al. (2017) to be tested under these conditions.

6.5 Future work and wider implications

There is clearly a great deal of work needed in order to fully understand the mechanisms driving inducible Si-defenses and Si uptake in tall fescue. Given its global importance as forage and turf grass (Hand et al., 2012a, b), and the importance of Si in helping plants resist both abiotic and biotic stresses (Guntzer et al., 2012), increased understanding of Si uptake in tall fescue is necessary. Sequencing of the genome would be beneficial to this process, as it would enable the sequences of genes involved in Si uptake to be found and would determine if single nucleotide polymorphisms exist in these transporters. Alternatively, proteomics of the sequences listed in McLarnon et al. (2017) would enable a better understanding of the proteins that are involved in inducible Si-based defenses. The results found here show a localised response to damage, in terms of Si accumulation, suggesting that Si is actively being redirected to these areas, the mechanisms behind this phenomenon remain unknown, but further investigation into the role of Lsi6, the leaf Si transporter may elucidate these. It is clear that the active transporter Lsi2 is important in Si uptake in the root, but understanding how Lsi6 is involved in inducible Si-based defences is crucial. The RNA-Seq results presented in this thesis identified potential Lsi6 sequences, which could be utilised by plant breeders for further investigation. In addition, preliminarily, unpublished work using an insect herbivore has shown that in mixed cultures of soft and harsh varieties, the soft varieties were eaten preferentially to the harsh varieties. Further investigation into the effect of harsh varieties and soft varieties on the wear of the herbivore mouthparts would be beneficial to elucidate the effectiveness of trichomes vs. phytoliths as anti-herbivore defenses in this species. Finally, further work is needed to determine how the structural components of the cell wall link to Si, especially in the context of inducible Si-based defenses.

The RNA-Seq work has provided a baseline for future molecular studies in tall fescue. Annotated sequences from the RNA-Seq work may be used to design primers for further study.

Specifically, more investigation into other defence related genes, such as those relating to jasmonic acid, could be investigated using the data generated in chapter 4, which was beyond the scope of the study, but would be beneficial in terms of understanding how Si supply affects defence mechanisms in this species and how this differs between undamaged and damaged plants.

Further, investigating the genes relating to the cell wall (such as cellulose and lignin related genes) in plants with high and low Si concentrations would be interesting, in terms of understanding how these traits are associated from a molecular perspective. This work may enable plant breeders to exploit selection for beneficial traits associated with high Si accumulating plants, but reduce the issues surrounding palatability.

The plant breeders may also utilise the molecular resources provided in this study (RNA-Seq and Si transporter sequences) to further insights into how the development of trichomes is related to Si-based defences.

Prior to the work presented in this thesis, molecular work on *F. arundinacea* was limited. This thesis has provided molecular resources (RNA-Seq), an annotated transcriptome for this species is now available, which can be exploited by plant breeders for targeted trait selection.

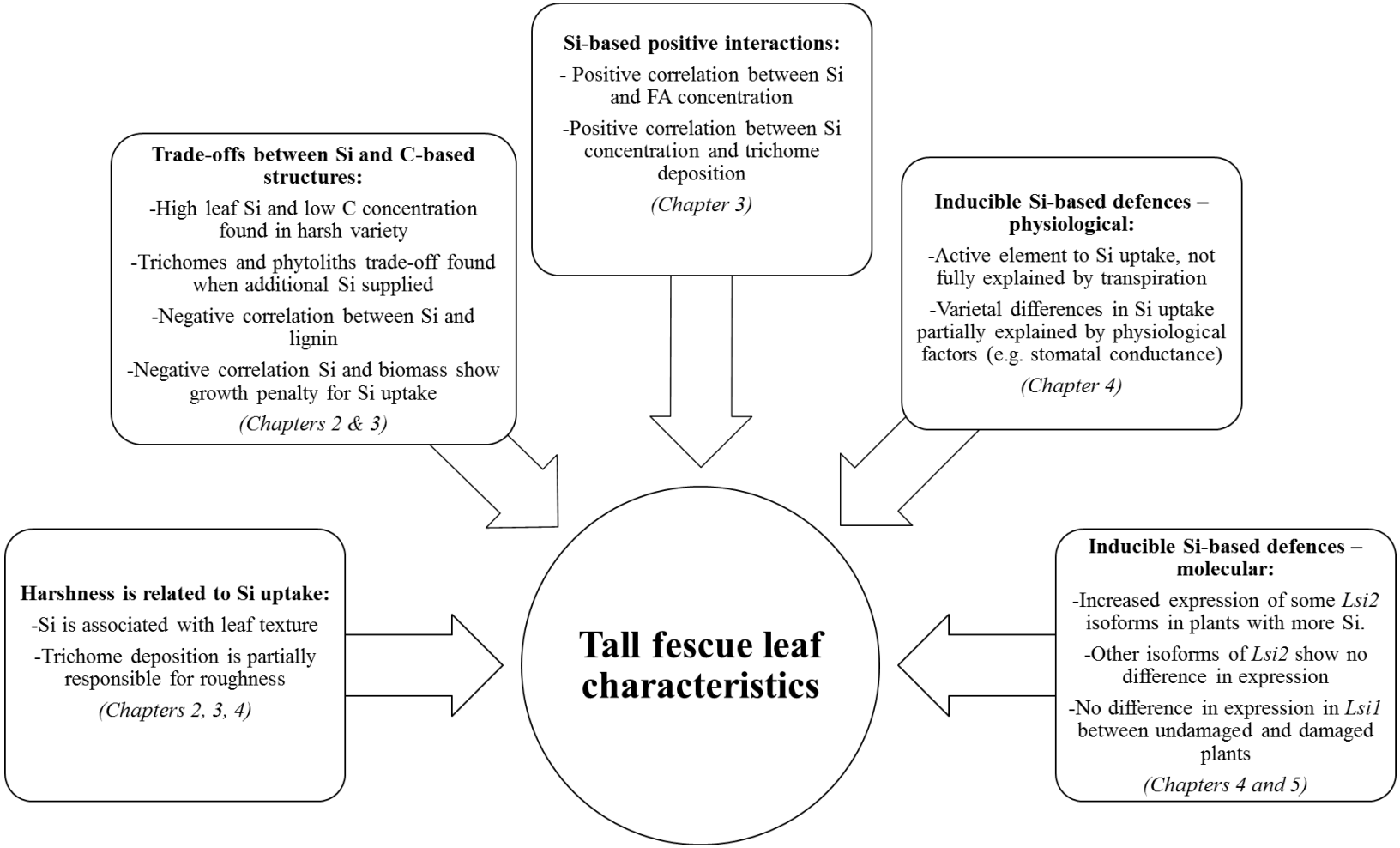

Figure 6.1: Main findings from the thesis. Abbreviations: Si = Silicon, C = Carbon, FA = Ferulic acid.

6.6 Conclusions

Tall fescue varieties are all able to accumulate Si, and they deposit Si into a range of structures, and it is the silicification of these structures which contributes towards leaf texture. These varietal differences in Si-based defenses are largely due to physiological factors, such as stomatal density. However, there is evidence of damage induced Si-based defenses in a number of varieties of tall fescue and have found evidence that harsh varieties are able to produce trichomes preferentially over phytoliths under high Si conditions, and this may be responsible for the harsh leaf textures reported. For the first time, this thesis also reports a potential trade-off within Si-based structures, indicating that these structures are costly to produce. It has also been found that Si may be used as a structural component and there is some evidence for trade-offs between Si and other leaf traits such as lignin content. Novel evidence shows that transpiration is an important driver in passive Si accumulation in this species, and may be partially responsible for varietal differences in Si uptake, but it cannot explain increased concentrations of Si in damaged plants. The thesis presents some novel evidence for changes in gene expression during induction of Si-based defenses, but more work is needed to fully understand the molecular basis of increases in Si after herbivory. The findings of this thesis have important implications for the selection of traits in plant breeding, and in understanding the complex mechanisms involved in Si uptake and deposition.

7 Abbreviations list

amp/ kan	Ampicillin/ kanamycin
ANOVA	Analysis of variance
Ar/ R	Aromatic/arginine selectivity filter
BBOT	2,2'-(2,5-Thienediyl)bis[5-(2-methyl-2-propanyl)-1,3-benzoxazole]
BLAST	Basic local alignment search tool
bp	Base pairs
C	Carbon
CDD	Conserved domain database
cDNA	complimentary DNA
D-	Undamaged plants
d.f.	Degrees of freedom
D+	Damaged plants
Dc	*Deschampsia cespitosa*
DFA	Diferulic acid
DGEA	Differential gene expression analysis
DM	Dry matter
DNA	Deoxyribonucleic acid
dT	Deoxythymine
DW	Dry weight
EDX	Energy dispersive x-ray spectroscopy
EeF1	Eukaryotic elongation factor 1
FA	Ferulic acid
Fo	*Festuca ovina*

Fr	*Festuca rubra*
Gb	Gigabase
GC	Guanine cytosine
G-S-G-R	Glycine - Serine -Glycine -Arginine
HCL	Hydrochloric acid
HPLC	High performance liquid chromatography
K	Potassium
LB	Lysogeny broth
logFC	log fold change
Mb	Megabase
N	Nitrogen
NCF	Neutral cellulose fibre
NDF	Neutral detergent fibre
NIP	Nodulin-26 like intrinsic proteins
NPA	Asparagine Proline Alanine
NS	Not significant
nts	Nucleotides
P	Phosphorous
pCA	p-Coumaric acid
PCR	Polymerase chain reaction
PIP	Plasma membrane intrinsic protein
P-XRF	Portable x-ray fluorescence
RNA	Ribonucleic acid
RNA-Seq	Ribonucleic acid sequencing
RSEM	RNA-Seq by Expectation Maximization
RT	Reverse transcriptase
RT-qPCR	Reverse transcription quantitative polymerase chain reaction

SE	Standard error
SEM	Scanning electron microscopy
Si	Silicon
SiO2	Silica
SIP	Small basic intrinsic protein
TFA	Trifluoroacetic acid
TIP	Tonoplastic intrinsic protein
Tm	Melting temperature
TMD	Transmembrane domain
TMHMM	Transmembrane helix markov model
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
XIP	Uncharacterised intrinsic protein

Varieties:

8	DLF-06-8
D	DLF-04-D
F	DLF-07-F
H	Harsh variety
J	DLF-05-J
K	DLF-13-K
M	DLF-10-M
N	DLF-14-N
S	Soft variety
SH	Semi-harsh variety
SS	Semi-soft variety
T	DLF-12-T
VS	Very soft variety

VVS Very very soft variety

8 References

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