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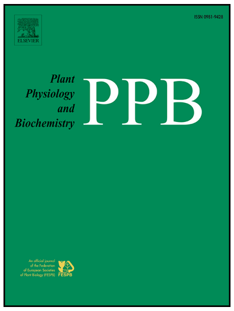

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Vacuolar Ca^{2+}/H^+ exchanger and Ca^{2+}-ATPase homologues are differentially regulated in tipburn-resistant and susceptible lettuce (*Lactuca sativa*) cultivars

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Abstract

Tipburn is a physiological disorder of lettuce (*Lactuca sativa*) and other leafy crops that causes external and internal leaf discolouration and results in serious quality issues for the fresh produce industry. Tipburn occurrence is difficult to predict and no completely effective control methods exist. This is compounded by poor knowledge of the underlying physiological and molecular basis of the condition, which appears to be associated with deficiency of calcium and other nutrients. Vacuolar calcium transporters, which are involved in calcium homeostasis in Arabidopsis, show differential expression in tipburn-resistant and susceptible *Brassica oleracea* lines. We therefore investigated expression of a subset of *L. sativa* vacuolar calcium transporter homologues, belonging to the Ca^{2+}/H^+ exchanger and Ca^{2+}-ATPase classes, in tipburn-resistant and susceptible cultivars. This indicated that some *L. sativa* vacuolar calcium transporter homologues belonging to these gene classes exhibited higher expression levels in resistant cultivars, whilst others had higher expression in susceptible cultivars or were independent of tipburn phenotype. In addition, some homologues were more highly expressed in symptomatic versus asymptomatic leaves in susceptible cultivars, suggesting that tipburn-induced increases in expression are unsuccessful in conferring resistance and that differential baseline expression of such genes is important for tipburn resistance. Knowledge of individual genes associated with tipburn resistance will improve breeding for such traits and the development of resistant lettuce varieties.

Keywords: Lettuce, *Lactuca sativa*, tipburn, calcium

1. Introduction

Tipburn, which is characterised by browning and collapse of leaf margins in fresh produce crops such as lettuce and brassicas, is an important quality issue for producers, processors and retailers. The condition occurs predominantly in the centre of the plant, either on externally visible or internal leaves. Its occurrence is hard to predict and to date no effective control measures exist.

Tipburn appears to be associated with deficiency of calcium and other nutrients (e.g. magnesium and potassium) (Collier and Wurr, 1981; Huett, 1994; Barta and Tibbetts, 2000; Birlanga et al., 2021). However, tipburn could instead be due to impaired calcium distribution within leaves, such as insufficient calcium delivery to leaf margins (Collier and Huntington, 1983; Hartz et al., 2007), for example due to insufficient transpirational flux.

Resistance to tipburn is a complex trait, subject to genetic (G) and environmental (E) influences and their interactive (GxE) effects (Jenni and Hayes, 2010). Determining the underlying molecular components of this trait is difficult due to the strong influence of growth conditions and plant morphology (Lee et al., 2016; Beacham et al., 2023). As such, identification of genomic contributors to tipburn resistance in lettuce has so far predominantly been limited to identification of quantitative trait loci (QTLs) in mapping populations (Jenni et al., 2013; Marcia-Gonzalez et al., 2019). The identification of specific *L. sativa* gene targets whose expression is associated with tipburn resistance or susceptibility is very limited (Macias-Gonzalez et al., 2021).

A small number of tipburn-associated genes are reported in other crops. Vacuolar calcium transporters such as the Ca^{2+}/H^{+} exchangers *CAX1* and *CAX3* and Ca^{2+}-ATPases *ACA4* and *ACA11*, which are involved in calcium homeostasis in Arabidopsis (Cheng et al., 2003; Lee et al., 2013), show differential expression in tipburn-resistant and susceptible *Brassica oleracea* lines (Lee et al., 2013) but their role in tipburn in *L. sativa* is unknown. Lettuce expressing deregulated Arabidopsis *CAX1* exhibited reduced sensitivity to calcium deficiency but the effect on tipburn was not investigated (Park et al., 2008). We therefore sought to determine the expression of *L. sativa CAX* and *ACA* homologues in resistant and susceptible lines to establish a possible role of calcium homeostasis via these genes in lettuce tipburn resistance.

Most studies of lettuce tipburn resistance have used a limited range of lettuce germplasm. We previously developed a commercially-relevant hydroponic assay to phenotype 96 lines of a *Lactuca* Diversity Fixed Foundation Set (DFFS) for tipburn resilience (Beacham et al., 2023). We identified significant ($p<0.001$) genotypic effects on tipburn resistance. Extreme lines from this earlier investigation were selected for analysis of tipburn-associated expression of vacuolar calcium transporter homologues in the present study. We expected to observe differential expression of such transporters between resistant and susceptible lines if they are involved in tipburn development.

2. Materials and Methods

2.1 Plant material and growing conditions

Plant material was generated in controlled glasshouse facilities (minimum day/night temperature of 15°C/5°C and supplemental lighting set at a 16h day where required) at Harper Adams University (Shropshire, UK) using a custom deep water hydroponic tipburn assay as per earlier studies (Beacham et al., 2023). Plants were grown initially in 345 module trays in Levington M2 compost then transplanted at the four true leaf stage into perlite and grown in an aerated 7 ml/l solution of Ionic HydroGrow (Growth Technology) nutrient mixture. Three previously-identified susceptible (cv. Ambassador, Verdatre and Alface) and three resistant (cv. Waldermann's Dark Green, Saladin and an unnamed *L. sativa* cultivar) DFFS lines were used. Two additional commercially-sourced cultivars of unknown tipburn resistance (cv. Electra and Themes) were included for comparison. Seed for lettuce DFFS lines was obtained from the UK Vegetable Genebank at the University of Warwick. Seed for cv. Electra and Themes were supplied by G's Growers (Ely, UK).Three plants per line were used. Tipburn was scored as previously (Beacham et al., 2023) from absent (0), through mild (1), moderate (3) to severe (5).

2.2 Gene expression and promoter analysis

Arabidopsis thaliana protein sequences translated from genes *AtCAX1* (NP_181352.1) *AtCAX3* (NP_190754.2), *AtACA4* (OAP07843.1) and *AtACA11* (OAP04734.1) were obtained from NCBI (https://www.ncbi.nlm.nih.gov/genbank/). Homologues in the *L. sativa* genome were identified through reciprocal BLASTp analysis (Altschul et al., 1990) of the *L. sativa* genome sequence at NCBI (Table 1).

Expression of *L. sativa* vacuolar calcium transporter homologues was determined in leaf margin samples from the tip and base of both tipburn-affected (if present) and symptomless leaves using RT-qPCR. Three samples were used per leaf location per symptom per lettuce line across three individual plants per line. RT-qPCR primers for detection of *L. sativa* vacuolar calcium transporter homologue expression were designed based upon the associated mRNA sequences of the *L. sativa* homologues (Table 1 and see Results section). Leaf samples were collected from plants co-temporally with tipburn phenotyping. Leaf samples were stored in RNAprotect Tissue Reagent (Qiagen) at 4°C until used. RNA was extracted from leaf samples using a RNeasy Plant Mini Kit (Qiagen). RNA concentration was determined using a NanoDrop spectrophotometer (ThermoFisher) and samples diluted to equal concentration. RT-qPCR was performed using a QuantiNova SYBR Green RT-qPCR Kit (Qiagen) and primers listed in Table 1. PCR parameters were used according to the manufacturer's instructions. *L. sativa* gDNA was used as a control and for calibration of RT-qPCR results. This was use to compare relative expression levels of the different genes (transcript relative abundance).

L. sativa genomic sequences were obtained from NCBI (https://www.ncbi.nlm.nih.gov). Potential *cis*-acting promoter elements were identified in 2 kb upstream genome regions of LOC111877411, LOC11191311 and LOC111890775 using plant CARE (Lescot et al., 2002, http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

2.3 Leaf calcium content determination

Leaf calcium content was determined for each cultivar from the pooled inner or outer whole leaves of the head by inductively coupled plasma optical emission spectroscopy (Onescientific, Bristol, UK). Samples were pooled to obtain sufficient material for analysis.

2.4 Data analysis

Data were analysed using ANOVA and Tukey's multiple comparison test at the 5% level in GenStat 20th Edition software (VSN International, Hemel Hempstead, UK). Graphs indicate mean values +/- one standard error of the mean (SEM).

Table 1. Target genes and primers used in this study.

***L. sativa* RNA ID**	**Associated *L. sativa* Gene ID**	**Arabidopsis Reciprocal Homology**	**Primer Name**	**Primer Sequence (5'-3')**
>XM_023873937.2	LOC111877411	CAX3, also CAX1	937F	TATGCAGCTGTTGCGAGTAC
			937R	CACAGCTGTTTCATCGTCAC
>XM_023908842.2	LOC111913111	'Cation exchanger 1'	842F	GATATGTTGGCAATGAGCC
			842R	TTCTTCTCTCACCTCCTCATC
>XM_023886862.2	LOC111890775	CAX3 and CAX1	862F	AACATGCTGTTTCACCTGTTG
			862R	ACCTTGTCATCCTTGGCCT
Multiple – referred to as 'ACA'		ACA4 and ACA11	ACAF	GAATCGCAGAAGTAGTCAACG
			ACAR	GACAATGGCGCATACTATAAGG

3. Results

3.1 Lettuce tipburn phenotype

Because of the strong environmental influence on tipburn development, the eight lettuce lines used in the study were phenotyped for tipburn symptom severity (Figure 1) in order to establish phenotype identity as expected from previous data (Beacham et al., 2023). This confirmed the susceptible (S) phenotype for Alface and Ambassador and resistant (R) phenotype for Saladin and Waldermann's Dark Green. Furthermore, the commercially-sourced cultivars Electre and Themes were both found to be resistant. However, the unnamed *L. sativa* cv. (previously resistant) and Verdatre (previously susceptible) exhibited an intermediate (I) phenotype. This may be due to a greater influence of growing environment on tipburn severity in these two lines.

3.2 Identification of L. sativa *Vacuolar Calcium Transporter Homologues*

Arabidopsis thaliana protein sequences for the vacuolar Ca^{2+}/H^{+} exchangers CAX1 (NP_181352.1) and CAX3 (NP_190754.2) and Ca^{2+}-ATPases ACA4 (OAP07843.1) and ACA11 (OAP04734.1) were used to search the *L. sativa* protein database for homologues. This revealed many similar results (29 for *CAX* genes and 100 for *ACA* genes) indicating a potentially large degree of redundancy among *CAX* and *ACA* genes in this species. The similarity between the Arabidopsis CAX1 and CAX3 sequences and between the ACA4 and

ACA11 sequences limited the ability to confidently identify *L. sativa* hits as homologues of one protein versus the other. Therefore, the three *L. sativa* hits with the highest degree of homology (measured by Expect value) to Arabidopsis CAX1 and/or CAX3 were selected for primer design based upon their associated mRNA sequences (Table 1). For the ACA protein hits, generic primers were designed to their corresponding RNA sequences with the aim of detecting expression of multiple similar *ACA* genes in lettuce (Table 1). Interestingly DNA melt curve analysis of products of *L. sativa* DNA amplification suggested a single product for all primer pairs.

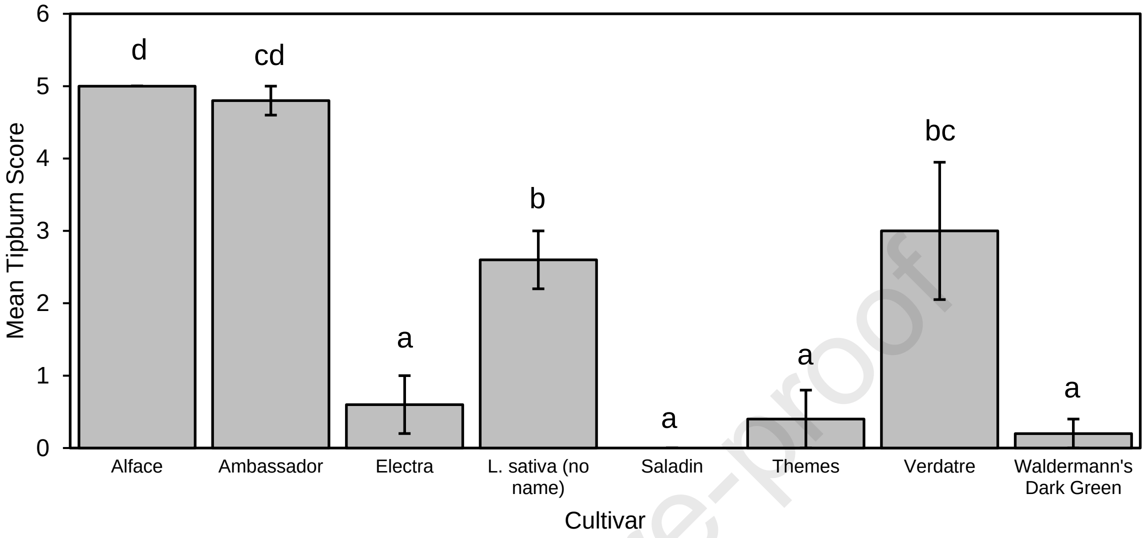


Figure 1. Tipburn phenotype of the eight lettuce cultivars studied. Tipburn severity was scored as in (Beacham et al., 2023). Lower tipburn scores indicate increased resistance. Bars indicate mean +/- SEM. Means which do not share any letters are significantly different (p<0.05).

3.3 *Expression of* L. sativa *Vacuolar Calcium Transporter Homologues*

A number of different factors were found to have significant effects on expression of the target genes in *L. sativa* samples (Table 2). For all four genes/gene groups, there was significant variation in expression between the different lettuce lines, between symptomless and tipburn-affected leaves ('leaf symptom' - with the exception of LOC111913111) and for LOC111890775 and ACA between leaf tip and base ('leaf part' - Table 2). Expression also varied significantly with tipburn phenotype (with the exception of LOC111890775). Most interactions between factors were non-significant.

Table 2. Factor influence on expression of *L. sativa* vacuolar calcium transporter homologues. Factors are: lettuce accession ('Line'), symptomless vs tipburn-affected leaves ('Leaf symptom'), leaf tip vs leaf base ('Leaf part') and tipburn phenotype (resistant, intermediate or susceptible, 'Phenotype'). Statistical significance is denoted as: *** - p<0.001, ** - p<0.01, * - p<0.05, ns – not significant.

	Gene ID			
Factor	**LOC111890775**	**LOC111877411**	**LOC111913111**	**ACA**
Line	**	***	***	***
Leaf symptom	***	***	ns	***
Leaf part	*	ns	ns	*
Phenotype	ns	***	**	***
Line x Leaf symptom	ns	***	**	***
Line x Leaf part	*	ns	*	ns
Leaf symptom x Leaf part	ns	ns	**	ns
Line x Leaf symptom x Leaf part	*	ns	ns	ns

Leaf symptom x Phenotype	ns	ns	ns	ns
Leaf part x Phenotype	ns	ns	ns	ns
Leaf symptom x Leaf part x Phenotype	ns	ns	ns	ns

The expression of the genes/gene groups was compared between lines exhibiting different tipburn phenotypes (Figure 2). This revealed that resistant lines exhibited significantly higher levels of expression of LOC111877411 and ACA compared to intermediate lines, with susceptible lines significantly lower still. Expression of LOC11191311, meanwhile, was significantly higher in susceptible lines compared to resistant or intermediate lines. For LOC111890775, there was no significant difference in expression between lines with different tipburn phenotypes. When this comparison is repeated with data from symptomless leaves only (data not shown), these relationships remain (with the exception that intermediate and susceptible lines are no longer significantly different for ACA expression). For symptomless leaves only (Table 3), there remained a significant variation in expression with lettuce line (except LOC111890775), leaf part (for LOC111890775 and LOC11191311) and phenotype (except LOC111890775). There were no significant interactions between factors for this dataset.

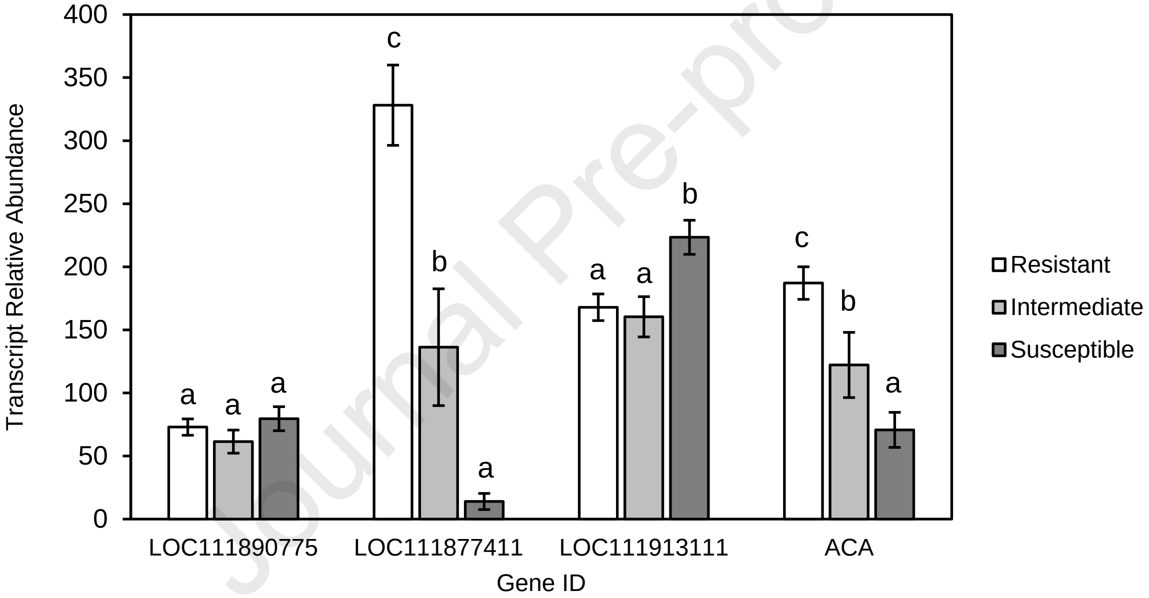


Figure 2. Influence of tipburn phenotype on relative transcript abundance of *L. sativa* vacuolar calcium transporter homologues in symptomless leaves only. Bars indicate mean +/- SEM. For each gene ID, means which do not share any letters are significantly different (p<0.05).

Table 3. Factor influence on expression of *L. sativa* vacuolar calcium transporter homologues in symptomless leaves only. Factors are: lettuce accession ('Line'), leaf tip vs leaf base ('Leaf part') and tipburn phenotype (resistant, intermediate or susceptible, 'Phenotype'). Statistical significance is denoted as: *** - p<0.001, ** - p<0.01, * - p<0.05, ns – not significant.

	Gene ID			
Line	**LOC111890775**	**LOC111877411**	**LOC111913111**	**ACA**
Line	ns	*	**	***
Leaf part	*	ns	*	ns
Phenotype	ns	***	**	***
Line x Leaf part	ns	ns	ns	ns
Leaf part x Phenotype	ns	ns	ns	ns

For the data from susceptible and intermediate lettuce lines only, there was a significant ($p<0.001$) effect of leaf symptom for LOC111890775, LOC111877411 and ACA (Figure 3). This indicates that in these lines, expression of these genes/gene groups is upregulated in leaves affected by tipburn symptoms compared to symptomless leaves. Expression of LOC111890775, which appears to be phenotype-independent was also up-regulated in affected leaves. Furthermore, there is no significant effect of leaf part for these three genes/gene groups, suggesting that symptom-responsive changes in gene expression occur throughout the margin of affected leaves, both at the tip (where symptoms frequently appear earlier and are more severe) and base. For LOC111913111, for which higher expression is associated with tipburn susceptibility, there was no significant overall response to the presence of tipburn symptoms but there was a significant overall ($p<0.05$) effect of leaf part with apparently greater expression in the tip versus the base of affected leaves.

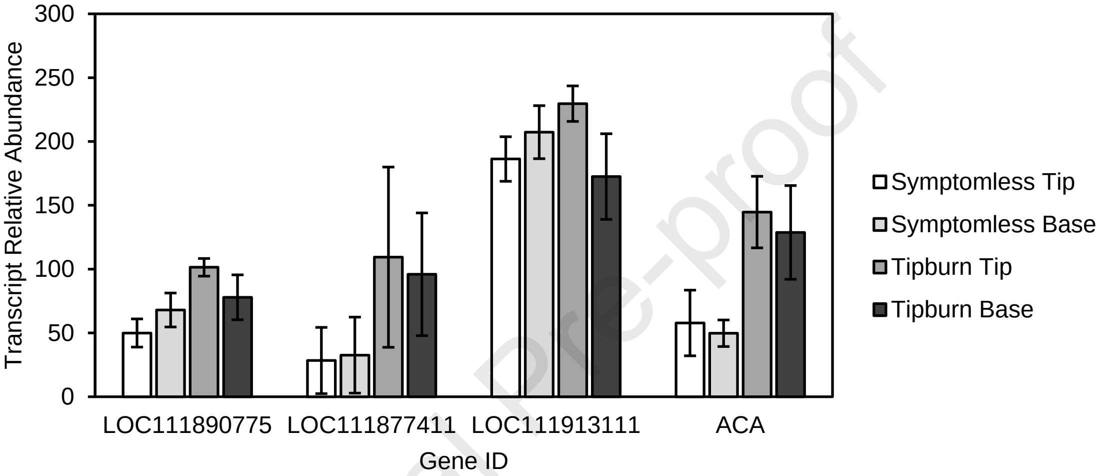


Figure 3. Influence of leaf symptom and leaf part on relative transcript abundance of *L. sativa* vacuolar calcium transporter homologues in leaves of susceptible and intermediate tipburn phenotype lines only. Bars indicate mean +/- SEM.

Analysis of the 5' upstream regions of the *L. sativa CAX* homologues LOC111877411, LOC11191311 and LOC111890775 revealed the presence of a number of potential *cis*-acting regulatory elements responding to stress, hormone and light-induced signals (Table 4).

3.4 Leaf Calcium Content

We did not find a strong correlation between mean tipburn severity and calcium content of inner ($r^2 = 0.07$) or outer ($r^2 = 0.36$) whole leaves.

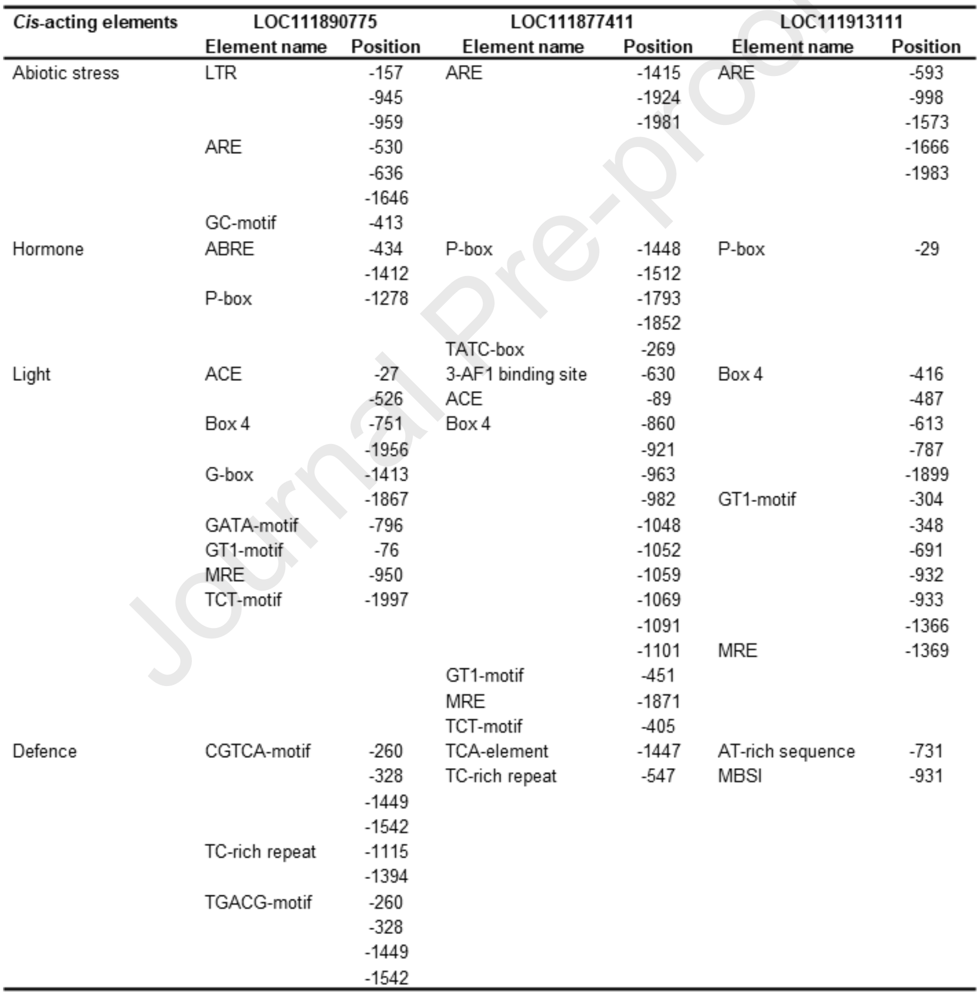
4. Discussion

These data indicate that different baseline expression levels of vacuolar Ca^{2+}/H^+ exchanger and Ca^{2+}-ATPase homologues are associated with different tipburn phenotypes, independent of symptom presence. This suggests that higher baseline expression of LOC111877411 and ACA and lower baseline expression of LOC111913111 are associated with tipburn resistance.

Given that higher expression of LOC111877411 and ACA are associated with tipburn resistance, the increase in expression of these genes/gene groups in tipburn-affected leaves may represent at attempt by the plant to combat the onset and/or development of tipburn. However, given that these lines do not exhibit resistance, this response appears to be ineffective, perhaps due to expression levels not reaching those found in resistant lines. Greater induced expression changes in response to tipburn occurrence, or alternatively greater differences in baseline (e.g. constitutive) expression of these genes/gene groups may be required for protection against tipburn. It is interesting to note that expression of LOC111890775 was also up-regulated in affected leaves yet independent of tipburn phenotype.

Identification of genetic contributors to tipburn resistance has, to date, relied mainly on the study of QTLs. Jenni et al. (2013) identified a QTL explaining 38-70% of the variance in tipburn incidence in an *L. sativa* cv. Emperor x cv. El Dorado mapping population, whilst Macias-Gonzalez et al., (2019) identified *qTPB5.2* and an additional major tipburn incidence QTL (*qTBIN_1_QC11*) in *L. sativa* cv. Salinas. *qTPB5.2* is located on linkage group (also chromosome) 5 of the lettuce reference map (Truco et al., 2013) whilst *qTBIN_1_QC11* is found on LG (also chromosome) 1. We further identified a small effect tipburn severity QTL in *L. sativa* cv. Iceberg, present on LG 12 (chromosome 1) (Beacham et al., 2023).

Table 4. Potential stress, hormone and light-responsive *cis*-acting regulatory elements in 2 kb upstream regions of *L. sativa CAX* homologues. Positions are given relative to the start of the ORF.



Cis-acting elements	LOC111890775		LOC111877411		LOC111913111	
	Element name	Position	Element name	Position	Element name	Position
Abiotic stress	LTR	-157	ARE	-1415	ARE	-593
		-945		-1924		-998
		-959		-1981		-1573
	ARE	-530				-1666
		-636				-1983
		-1646				
	GC-motif	-413				
Hormone	ABRE	-434	P-box	-1448	P-box	-29
		-1412		-1512		
	P-box	-1278		-1793		
				-1852		
			TATC-box	-269		
Light	ACE	-27	3-AF1 binding site	-630	Box 4	-416
		-526	ACE	-89		-487
	Box 4	-751	Box 4	-860		-613
		-1956		-921		-787
	G-box	-1413		-963		-1899
		-1867		-982	GT1-motif	-304
	GATA-motif	-796		-1048		-348
	GT1-motif	-76		-1052		-691
	MRE	-950		-1059		-932
	TCT-motif	-1997		-1069		-933
				-1091		-1366
				-1101	MRE	-1369
			GT1-motif	-451		
			MRE	-1871		
			TCT-motif	-405		
Defence	CGTCA-motif	-260	TCA-element	-1447	AT-rich sequence	-731
		-328	TC-rich repeat	-547	MBSI	-931
		-1449				
		-1542				
	TC-rich repeat	-1115				
		-1394				
	TGACG-motif	-260				
		-328				
		-1449				
		-1542				

Macias-Gonzalez et al. (2021) recently dissected *qTPB5.2* and detected differential expression between tipburn resistant and susceptible lines for two Ca^{2+}/Na^{+} exchanger homologue encoding genes (Lsat_5X129180, aka LOC111919988 and Lsat_5X129040, aka LOC111919987), located 21 kb apart on chromosome 5. The Ca^{2+}/H^{+} exchanger homologue encoding genes LOC111890775 and LOC111913111 from this study are also found on chromosome 5 but do not co-locate, being positioned 30 Mb and 61 Mb distant, respectively. LOC111877411, meanwhile, is located on chromosome 8, where Jenni et al. (2013) identified additional tipburn resistance QTLs. It will be interesting to determine whether the genes investigated in this study are found within the limits of these QTLs identified previously.

Whilst Arabidopsis CAX1 localises to the tonoplast (Cheng et al., 2003), interestingly, the protein encoded by the Arabidopsis orthologue of Lsat_5X129180 (At1g53210, aka *AtNCL*), whilst involved in calcium homeostasis, is found instead in the plasma membrane (Wang et al., 2012). Future studies will aim to determine the subcellular localisation of the genes identified in this study and whether calcium flux across the plasma membrane and/or tonoplast may be involved in tipburn resistance. Expression and activity of different classes of plant calcium transport proteins appears to be interlinked. The Arabidopsis *cax1* mutant exhibits not only increased expression of fellow Ca^{2+}/H^+ exchanger *CAX* genes but also reduced tonoplast V-type H^+-translocating ATPase and increased tonoplast Ca^{2+}-ATPase activity (Cheng et al., 2003). This may help to explain the association of both *CAX* and *ACA* genes with tipburn phenotype found in this study.

Furthermore, Macias-Gonzalez et al. (2021) found allelic differences in Lsat_1_v5_gn_5_129180 courtesy of a single nucleotide polymorphism which correlated with tipburn phenotype. Sequencing of the genes from this study across the cultivars of the DFFS will allow detection of alleles associated with tipburn resistance and provide further mechanistic insight to development of this condition.

We found that changes in expression of LOC111877411 and ACA in response to tipburn development occurred both at the tip margin, where calcium deficiency could be more impacted by transpirational flux and at the base margin. LOC111913111, whose expression was negatively related to tipburn resistance, exhibited greater expression in the tip versus the base of affected leaves but no influence of symptom development. This difference could contribute to a greater degree of symptom severity in the tip versus the base of affected leaves but will require further investigation that considers the effects of additional factors such as increased transpiration stream distance. Because of sampling necessities, Macias-Gonzalez et al., (2021) were unable to differentiate expression between affected and symptomless leaf parts or between leaf tip and base. The data presented here suggest changes in expression of calcium homeostasis-associated genes in affected *L. sativa* leaves upon symptom occurrence and between leaf tip and base the warrant further investigation.

Together, with the reduced sensitivity to calcium deficiency displayed by lettuce expressing deregulated Arabidopsis *CAX1* (Park et al., 2008), these studies further suggest the importance of calcium homeostasis in tipburn resistance. Additional research will help to further determine the roles of calcium subcellular partitioning, and its effects on cell wall integrity (Tibbitts et al., 1965; Olson et al., 1967) and signalling (Cheng et al., 2003) in tipburn resistance in this and other species.

We did not find a strong correlation between mean tipburn severity and calcium content of whole inner ($r^2 = 0.07$) or outer ($r^2 = 0.36$) lettuce leaves, as has been noted in some *B. oleracea* lines (Lee et al., 2016). However, the distribution of calcium within individual leaves,and perhaps in tissues and between cellular compartments, also appears to play a role in tipburn resistance. Within an affected lettuce leaf, the areas exhibiting symptoms have been shown to contain lower calcium concentrations than unaffected parts of the same leaf (Barta and Tibbitts, 1991). Similarly, Lee et al (2016) found *B. oleracea* to exhibit higher calcium content in the leaf base compared to the leaf apex. In this study we were not able to quantify localised calcium levels at the edge of leaves as it was necessary to pool samples across multiple whole leaves in order to obtain sufficient material for analysis. Future studies will investigate the distribution of calcium within leaves and its relationship to the transcriptome of *L. sativa* in order to more fully investigate gene expression profiles in tipburn resilient and susceptible lines.

The presence of potential *cis*-acting regulatory elements responding to stress, hormone and light-induced signals in the 5' upstream regions of the *L. sativa CAX* homologues LOC111877411, LOC11191311 and LOC111890775 is comparable to previous findings for *B. oleracea CAX* and *ACA* genes (Lee et al., 2016) and suggests interlinking between calcium homeostasis and regulation of hormonal and stress responses, which may also be involved in tipburn resistance in *L. sativa* and indeed, whose associated genes are differentially regulated between tipburn resistant and susceptible lines in *B. oleracea* (Lee et al., 2016).

The unpredictability and limited molecular understanding of tipburn necessitate identification of genes involved in the occurrence and severity, and therefore resistance to, this condition. We have identified differential expression of Ca^{2+}/H^+ exchanger and Ca^{2+}-ATPase-encoding genes between tipburn-resistant and susceptible lettuce lines, which have not been previously linked to tipburn in this species. Some of these genes were more highly expressed in symptomatic versus asymptomatic leaves in susceptible cultivars, suggesting that tipburn-induced increases in gene expression are unsuccessful in conferring resistance. Rather, data suggest that differential baseline expression of such genes is important for tipburn resistance. These data may aid the development of new varieties with improved baseline expression profiles and enhanced tipburn resilience. Further investigation of the link between calcium transporter expression, including elucidating the individual *ACA* gene expression profiles and subcellular protein and calcium distribution will also further this aim.

Author's Contributions

A Beacham applied for funding with grant proposal editing from K Wilkins, J Davies and J Monaghan. A Beacham and J Monaghan performed the gene expression work. K Wilkins performed the calcium content work. A Beacham analysed the data and wrote the manuscript. J Monaghan edited the manuscript. J Davies and J Monaghan provided supervisory assistance. Vertical future assisted with the calcium content work and funding for K Wilkins.

Declaration of competing interest

The authors declare no known competing financial interests or personal relationships that would influence the work reported in this paper. Financial support for K Wilkins was partly assisted by Vertical Future. G's Growers Ltd provided two lettuce lines.

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***Cis*-acting elements**	**LOC111890775**		**LOC111877411**		**LOC111913111**	
	Element name	**Position**	**Element name**	**Position**	**Element name**	**Position**
Abiotic stress	LTR	-157	ARE	-1415	ARE	-593
		-945		-1924		-998
		-959		-1981		-1573
	ARE	-530				-1666
		-636				-1983
		-1646				
	GC motif	-413				
Hormone	ABRE	-434	P-box	-1448	P-box	-29
		-1412		-1512		
	P-box	-1278		-1793		
				-1852		
			TATC-box	-269		
Light	ACE	-27	3-AF1 binding site	-630	Box 4	-416
		-526	ACE	-89		-487
	Box 4	-751	Box 4	-860		-613
		-1956		-921		-787
	G-box	-1413		-963		-1899
		-1867		-982	GT1-motif	-304
	GATA-motif	-796		-1048		-348
	GT1-motif	-76		-1052		-691
	MRE	-950		-1059		-932
	TCT-motif	-1997		-1069		-933
				-1091		-1366
				-1101	MRE	-1369
			GT1-motif	-451		
			MRE	-1871		
			TCT-motif	-405		
Defence	CGTCA-motif	-260	TCA-element	-1447	AT-rich sequence	-731
		-328	TC-rich repeat	-547	MBSI	-931
		-1449				
		-1542				
	TC-rich repeat	-1115				
		-1394				
	TGACG-motif	-260				
		-328				
		-1449				
		-1542				

Highlights:

- Ca^{2+}/H^{+} exchangers and Ca^{2+}-ATPases differentially regulated in tipburn-resistant and susceptible lines.
- Increased expression of some of these genes associated with resistance, of others with susceptibility.
- Tipburn-induced increases in expression are unsuccessful in conferring resistance.
- Tipburn resistance is instead associated with differences in baseline expression levels.

Contribution

- A Beacham applied for funding with grant proposal editing from K Wilkins, J Davies and J Monaghan
- A Beacham and J Monaghan performed the gene expression work.
- K Wilkins performed the calcium content work.
- A Beacham analysed the data and wrote the manuscript. J Monaghan edited the manuscript.
- J Davies and J Monaghan provided supervisory assistance.
- Vertical future assisted with the calcium content work and funding for K Wilkins.

Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

K Wilkins reports financial support was provided by Vertical Future. A Beacham reports equipment, drugs, or supplies was provided by G's Growers Ltd. K Wilkins reports a relationship with Vertical Future that includes: employment.