



# Identification of novel QTLs for salt tolerance in zombi pea (*Vigna vexillata*)

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**Abstract** Soil salinity is a major abiotic stress in agricultural production, as it hampers the growth and development of crop plants. Developing crop cultivars with salinity/salt tolerance is a major goal in many plant breeding programs. This study aimed to identify quantitative trait locus (QTL) that controls salt tolerance in the wild zombi pea (*Vigna vexillata*) accession “AusTRCF 322105.” For QTL analysis, a BC<sub>1</sub>F<sub>2</sub> population resulting from the cross between salt-susceptible TVNu240 and TVNu240 × Aus-TRCF 322105 was used. A genetic linkage map was constructed for the BC<sub>1</sub>F<sub>2</sub> population, consisting of 15 linkage groups and using 296 SNP markers. The map spanned 2889.9 cM in total length. Plant leaf wilt and plant survival were evaluated by subjecting the BC<sub>1</sub>F<sub>2</sub> population to a hydroponic condition with 250 mM NaCl to assess salt tolerance at the seedling stage. QTL analysis revealed that two QTLs, *qSaltol\_3.1* and *qSaltol\_7.1*, control both leaf wilt and

plant survival. These two QTLs explained 23–27% and 11–15% of the trait variation. Exploration of the physical genome region revealed that *qSaltol\_3.1* is located near genes encoding methionine sulfoxide reductase and protein L-isoaspartate O-methyltransferase that are involved in oxidative stress resistance. In contrast, *qSaltol\_7.1* is located near genes encoding 3-ketoacyl-CoA synthase, L-type lectin domain containing receptor kinase VII.1, lipoxygenase 3, CBS domain-containing protein 5, NAC25 protein, and asparagine synthetase (ASNS) that have been found to be associated with salt tolerance. Nonetheless, *qSaltol\_3.1* and *qSaltol\_7.1* are novel QTLs identified for salt tolerance in the zombi pea.

**Keywords** *Vigna vexillata* · Salt tolerance · QTL · Zombi pea · Segregation distortion

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## Introduction

Abiotic stresses such as high temperature, high CO<sub>2</sub>, drought, flood, acidic soil, alkaline soil, saline soil, and nutritional deficiencies are environmental stresses that can adversely affect the growth, development, and yield of crop plants. These stresses are expected to worsen in the near future due to climate change. Soil salinity is one of the most important abiotic stresses in crop production (Yamaguchi and Blumwald 2005; Shahbaz and Ashraf 2013). Soil salinity refers to the presence of excess water-soluble

salts, including sodium ( $\text{Na}^+$ ), potassium ( $\text{K}^+$ ), chloride ( $\text{Cl}^-$ ), and sulfate ( $\text{SO}_4^{2-}$ ), especially  $\text{Na}^+$ , in the soil (Stavi et al. 2021). The occurrence and increment of soil salinity can be due to various factors, including irrigation with saline water, natural weathering processes, and sea level rise (Stavi et al. 2021). It has been estimated that the amount of world agricultural land destroyed by salt accumulation is about 10 mha per annum (Pimentel et al. 2004), and the saline soil affects approximately 20% (45 mha) of the world's cultivated area (Shrivastava and Kumar 2015).

In plant systems, salt affects plants by (i) reducing water potential that results in osmotic stress (Munns 2005; Shrivastava and Kumar 2015), (ii) inducing ion toxicity, particularly  $\text{Na}^+$  and  $\text{Cl}^-$ , due to the excess accumulation of ions in plant cells (Jamil et al. 2011; Machado and Serralheiro 2017), and (iii) disrupting ion imbalance or disrupting ion homeostasis that results in nutrient imbalance (Jamil et al. 2011; Shrivastava and Kumar 2015). These effects eventually lead to growth inhibition and yield losses or even kill the plants (Mishra et al. 2023; Yamaguchi and Blumwald 2005). Scientists have long been interested in developing efficient and reliable approaches to crop production in saline soils (Munns 2005). Using salt-tolerant cultivars is among the most efficient approaches for sustainably managing salt-affected soils (Mishra et al. 2023). Developing salt-tolerant cultivars is a primary objective of many breeding programs. However, domesticated crops are generally susceptible to abiotic stresses such as saline soil. Therefore, to develop salt-tolerant cultivars/varieties, wild plant genetic resources must be exploited for useful genes conferring salt tolerance (Yamaguchi and Blumwald 2005; Mba et al. 2012; Lebot 2013). For example, the wild tomato *Solanum pimpinellifolium* has been found to possess tolerance to salt, and the tolerance gene in this wild species was used to improve salt tolerance in a cultivated tomato (*S. lycopersicum*) (Razali et al. 2018). Nonetheless, salt tolerance is a complex quantitative trait. A detailed understanding of the molecular mechanisms underlying salt tolerance is necessary and useful for improving the salt tolerance of crop plants using biotechnological methods.

*Vigna* crops, including mung bean (*Vigna radiata* (L.) R. Wilczek), black gram (*Vigna mungo* (L.) Hepper), rice bean (*Vigna umbellata* (Thunb.) Ohwi and Ohashi), azuki bean (*Vigna angularis* (Ohwi) Ohwi

and Ohashi), moth bean (*Vigna aconitifolia* (Jacq.) Maréchal), cowpea (*Vigna unguiculata* (L.) Walp.), Bambara groundnut (*Vigna subterranea* (L.) Verdc.), zombi pea (*Vigna vexillata* (L.) A. Rich), and créole bean (*Vigna reflexo-pilosa* Hayata) are important tropical leguminous crops grown in more than 25 mha in Africa, Asia, Australia, and America (Somta et al. 2009). They are grown mainly by small-land holders for dry seeds with a protein content of about 20–25% and a carbohydrate content of about 60–65% and are used as foods in various ways (Tomooka et al. 2002, 2009). Like other legume crops, these *Vigna* crops are generally susceptible to salt stress. However, wild *Vigna* species, including wild mung bean (*V. radiata* var. *sublobata*), wild zombi pea, *Vigna nakashimae* (Ohwi) Ohwi & Ohashi, *Vigna riukiensis* (Ohwi) Ohwi & Ohashi, *Vigna trilobata* (L.) Verdc., *Vigna luteola* (Jacq.) Benth., and *Vigna marina* (Burm.) Merr. are found growing naturally under saline soil or saline-associated environment (Lawn and Cortell 1988; Tomooka et al. 2002, 2009; Lawn and Watkinson 2002; Karuniawan et al. 2006). Some germplasm accessions of these wild *Vigna* species have been identified as moderately or highly tolerant to salt stress (Chankaew et al. 2014; Iseki et al. 2016; Dachapak et al. 2019; Yoshida et al. 2020; Noda et al. 2022). A few studies have identified quantitative trait loci (QTL) controlling salt tolerance in the wild *Vigna* species, including *V. marina* (Chankaew et al. 2014), *V. vexillata* (Dachapak et al. 2019), *V. nakashimae*, and *V. riukiensis* (Ogiso-Tanaka et al. 2023). These QTLs can be used to enhance salt tolerance in *Vigna* through intra- and interspecific hybridization in combination with marker-assisted selection (MAS). MAS, especially marker-assisted backcrossing (MABC), is a promising strategy for developing new crop cultivars with reduced time and cost while increasing precision (Collard and McKill 2008).

Among the domesticated *Vigna* species, the zombi pea is an interesting species. The cultivated zombi pea is an underutilized crop grown in limited areas of Africa and Asia. There are two types of cultivated zombi peas: seed and tuberous. Molecular genetic diversity analysis of the zombi pea showed that the seed type was domesticated in Africa, while the tuberous type was domesticated in Asia (Dachapak et al. 2017). The wild zombi pea is highly diverse, and up to seven botanical varieties have been identified for this species. Wild zombi peas can be found

in tropical and subtropical regions of Asia, Africa, and America. The diversity of this species can be exploited to improve the genetics of *Vigna* species. However, studies on the genetics of useful traits for genetic improvement in zombi peas are scarce. Up to the present, there is only one study on the genetics of salt tolerance in this species. Dachapak et al. (2019) reported a QTL mapping study for salt tolerance at 250 mM NaCl under hydronic conditions in wild zombi pea accession “JP235908” (var. *ovata*). They identified three QTLs for the tolerance, with each QTL explaining between 8.1% and 13.3% of the salt tolerance variation.

This study reported the QTLs controlling salt tolerance in a new source of salt tolerance wild of zombi pea, accession “S261” (var. *ovata*). Comparative genome analysis revealed that the QTLs identified for salt tolerance in accession S261 are novel.

## Materials and methods

### Plant materials and DNA extraction

This study used a BC<sub>1</sub>F<sub>2</sub> population comprising 93 plants as a mapping population. The population was derived from the crossing of zombi pea accessions “TVNu 240” and “AusTRCF 322105.” The accession TVNu 240 is a cultivated form (*V. vexillata* var. *macroserma*) from the Central African Republic and is susceptible to salinity stress (Dachapak et al. 2019). In contrast, AusTRCF 322105, hereafter called “S261” is a wild zombie pea (*V. vexillata* var. *vexillata*) from Australia and is tolerant to salinity stress (Fig. 1). An F<sub>1</sub> hybrid plant obtained from a cross between TVNu240 and S261 was grown and self-pollinated to generate the F<sub>2</sub> population. After that, F<sub>2</sub> and parental seeds germinated, and the resulting plants were self-pollinated. The F<sub>3</sub> seeds from each plant were harvested separately for the subsequent evaluation of salt tolerance using the procedures described in the “salt stress evaluation.” Based on the evaluation of salt tolerance, an F<sub>2,3</sub> line/family that all the plants were highly resistant was selected, and a single plant of the selected line was then backcrossed with TVNu240 to produce the BC<sub>1</sub>F<sub>1</sub> population. The BC<sub>1</sub>F<sub>1</sub> population was subjected to salt tolerance evaluation (see below), and a single BC<sub>1</sub>F<sub>1</sub> plant showing high salt tolerance was selected, transferred to normal soil, and



**Fig. 1** Response of cultivated zombi pea (TVNu240) and wild zombi pea (S261) plants grown in a nutrient solution containing 250 mM NaCl for 21 days

self-pollinated to generate the BC<sub>1</sub>F<sub>2</sub>. Subsequently, BC<sub>1</sub>F<sub>2</sub> plants and their parental plants were grown under field conditions at Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, Thailand, and then self-pollinated. The BC<sub>1</sub>F<sub>2,3</sub> seeds from each plant were harvested separately for the subsequent evaluation of salt tolerance.

The total genomic DNA of each BC<sub>1</sub>F<sub>2</sub> plant and the parental plants was extracted from the young leaves of each plant using a modified CTAB method given by Lodhi et al. (1994). The DNA quality and quantity were assessed on 0.8% agarose gel electrophoresis and a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). The DNA was diluted to 20 ng/μl for DNA library construction.

### Salt stress evaluation

BC<sub>1</sub>F<sub>3</sub> seeds from each BC<sub>1</sub>F<sub>2</sub> plant were sown in trays with peat moss for 10 days. Then BC<sub>1</sub>F<sub>3</sub> plants derived from each BC<sub>1</sub>F<sub>2</sub> plant were transplanted to

a hydroponic culture in a greenhouse of the Department of Agronomy, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University, Kamphaeng Saen Campus, Thailand. The nutrient solution used for hydroponic culture in this study was a modified Hoagland solution (Srinives et al. 2010). Seven days after transplanting, the nutrient solution containing 150 mM NaCl was used for the hydroponic culture for seven days, and then the NaCl concentration was increased by 50 mM at seven-day intervals until a final concentration of 250 mM was reached. After that, the NaCl concentration was maintained at 250 mM for 21 days. Seven-day intervals after applying the highest concentration of 250 mM NaCl solution, the leaf wilt of each plant was recorded. Leaf wilt was visually scored using scales of 1–5, where 1 = normal healthy leaves, 2 = 1–25% of leaves wilted, 3 = 26–50% of leaves wilted, 4 = 51–75% of leaves wilted, and 5 = 76–100% of leaves wilted or plant completely dead (Supplementary Fig. 1). Each plant was scored by three trained staff, and the average score was used for data analysis. In addition, at each time of leaf wilt scoring, the number of survival plants in each line was counted and converted into a percentage. Parental plants were also included in the salt tolerance evaluation.

The leaf wilt scores (LWS) and percentage of survival plants (PSP) were calculated for the area under the disease progress curve (AUDPC) following Simko and Piepho (2012). AUDPC is an indicator of the progression of the saline severity in this study. The AUDPC value of leaf wilt scores and the percentage of survival plant of each BC<sub>1</sub>F<sub>3</sub> plant were used for further analyses.

#### Genotyping-by-sequencing (GBS) analysis

A multiplexed GBS library was prepared using the modified GBS protocol described by Mascher et al. (2013). Briefly, the DNA sample of each plant was digested with two enzymes (*Pst*I/*Msp*I). Adapters compatible with the primers of the Ion Torrent Proton sequencing platform; the forward adapters contained 9-bp unique barcodes in addition to 21 bp of the Ion Forward adapter and a *Pst*I restriction site; and the reverse adapter (Y-adapter) contained the Ion reverse priming site and was ligated to digested DNA fragments. The final PCR-amplified library fragments were examined for size distribution using

the BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA), and the prevalence of products was observed to be between 100 and 200 bp. In order to take full advantage of the Ion PI™ Template OT2 200 Kit (Life Technologies, Grand Island, NY, USA), which supports 200-base read libraries, size-selected fragments of ~270 bp (the combined length of the forward and reverse adapter sequences was ~70 bp) using the E-Gel® SizeSelect™ Agarose Gels (Life Technologies, Grand Island, NY, USA) were performed. The libraries were subsequently quantified using the 2100 Bioanalyzer High Sensitivity DNA kit (Agilent Technologies, Santa Clara, CA, USA) and diluted to 100 pM for emulsion PCR amplification. The libraries were sequenced on the Ion Proton PI™ Chips according to the manufacturer's protocol (Life Technologies, Grand Island, NY, USA).

#### Sequence data analysis and SNP identification

Raw reads were de-multiplexed according to their barcodes, and the adapter/barcode sequences were removed using the standard Ion Torrent™ Suite software. Clean reads were mapped to the *V. vexillata* reference genome (Naito et al. 2022) using the Ion Torrent™ Suite Software Alignment Plugin (Torrent Mapping Alignment Program Version 4.0.6), and the variants were called using the Ion Torrent Variant Caller (GATK v1.4–749-g8b996e2; Life Technologies, Grand Island, NY, USA). The following (default) parameter setting was applied: minimum sequence match on both sides of the variants of 5, minimum support for a variant to be evaluated of 6, minimum frequency of the variant to be reported of 0.15, and maximum relative strand bias of 0.8. The uniformity of base coverage was determined using the Ion Torrent Suite Software Coverage Analysis Plugin (Life Technologies, Grand Island, NY, USA), and it was defined as the percentage of bases in all targeted regions (or whole genome) covered by at least 0.2× the average base coverage depth. The types of mutations from the GBS data caused by single nucleotide variations in the coding regions were analyzed using the software SnpEff (Cingolani et al. 2012) with the *V. vexillata* reference genome sequence (Naito et al. 2022) and GFF annotation input files.

## Genetic linkage map construction and QTL identification

A genetic linkage map was constructed for the BC<sub>1</sub>F<sub>2</sub> population using the software QTL IciMapping 4.2 (Meng et al. 2015). The markers were grouped with a logarithm of the odds (LODs) value of 5.0. The orders of the markers in the linkage group were determined by the REcombination Counting and ORDERing algorithm (Van Os et al. 2005) and rippled by the Sum of Adjacent Recombination Frequencies function (Falk 1989). The genetic distance in centimorgan units (cM) between the markers was calculated using the Kosambi mapping function.

The location of the QTLs was determined by inclusive composite interval mapping (ICIM) (Li et al. 2007) using the same software for a linkage analysis. The AUDPC value of leaf wilt scores and the percentage of survival plants in the 93 lines used to construct the linkage map are used to locate QTLs associated with salt tolerance. ICIM was performed at every 0.1 cM using a probability in stepwise regression of 0.001. LOD score of 3.0 was used as significant LOD threshold for QTLs for each trait.

## Segregation distortion analysis

The segregation ratios of the markers in the population were examined by chi-square goodness-of-fit analysis. The markers whose segregation ratio deviated from the expected 1:2:1 ratio at  $P < 0.05$  were classified as distorted markers.

## Results

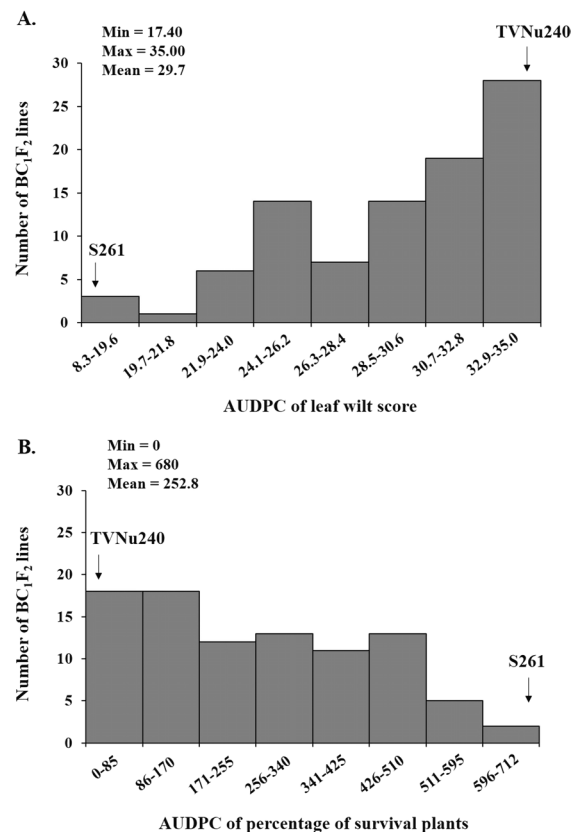
### Salinity tolerance variation in the BC<sub>1</sub>F<sub>2</sub> population

The LWS and PSP of 93 BC<sub>1</sub>F<sub>2</sub> lines (10 BC<sub>1</sub>F<sub>3</sub> plants per line) and the parents (TVNu240 and S261) were evaluated after subjecting the plants to a nutrient solution containing 250 mM NaCl. The AUDPC calculated for the LWS and PSP was used to measure the tolerance to salt stress. The parents responded contrastingly to the salt stress (Fig. 1). The AUDPC of leaf wilt scores for TVNu240 and S261 were 35 and 9.6, respectively, whereas that of the BC<sub>1</sub>F<sub>2</sub> population varied between 17.4 and 35.0 with an average of 29.7. The AUDPC of LWS showed continuous

distribution and skewed towards TVNu240 (Fig. 2A). The AUDPC of PSP for TVNu240 and S261 were 0 and 711.3, respectively. The AUDPC of PSP in the BC<sub>1</sub>F<sub>2</sub> population ranged between 0 and 680, with a mean of 252.8. This trait also exhibited a continuous distribution (Fig. 2B).

### SNP discovery and genotyping by GBS and linkage map construction

SNP marker genotyping of the BC<sub>1</sub>F<sub>2</sub> population was conducted by GBS. In total, 472 Gbp were obtained for the BC<sub>1</sub>F<sub>2</sub> population, with an average of 4.92 Gbp per individual; 96.02% of the bases were high quality (>Q30), and the average GC content was 39.23%. The total number of SNPs in the population



**Fig. 2** Frequency distributions of AUDPC of leaf wilt score and AUDPC of percentage of survival plants in the BC<sub>1</sub>F<sub>2</sub> population subjected to salinity stress at 250 mM NaCl. The BC<sub>1</sub>F<sub>2</sub> population is derived from the cross TVNu240 × (TVNu240 × S261)

was 552,470 loci, which were then filtered to 19,050 loci and used in the linkage analysis.

Among the 19,050 SNP loci, redundant loci were removed. Finally, a total of 296 SNPs were used for constructing the linkage map. A linkage map comprised of 15 linkage groups (LGs 1–15) was constructed (Fig. 3; Table 1). The map covered a total genetic distance of 2,889.90 cM, with the distance of each linkage group ranging from 30.06 cM in LG9 to 408.74 cM in LG2 (Table 1). The number of SNP markers mapped in each linkage group varied from 3 markers (LG9 and 15) to 43 markers (LG7), with an average of 19.73 SNPs per linkage (Table 1). The average marker spacing was 9.76 cM, ranging from 6.67 cM in LG6 to 12.97 cM in LG8 (Table 1).

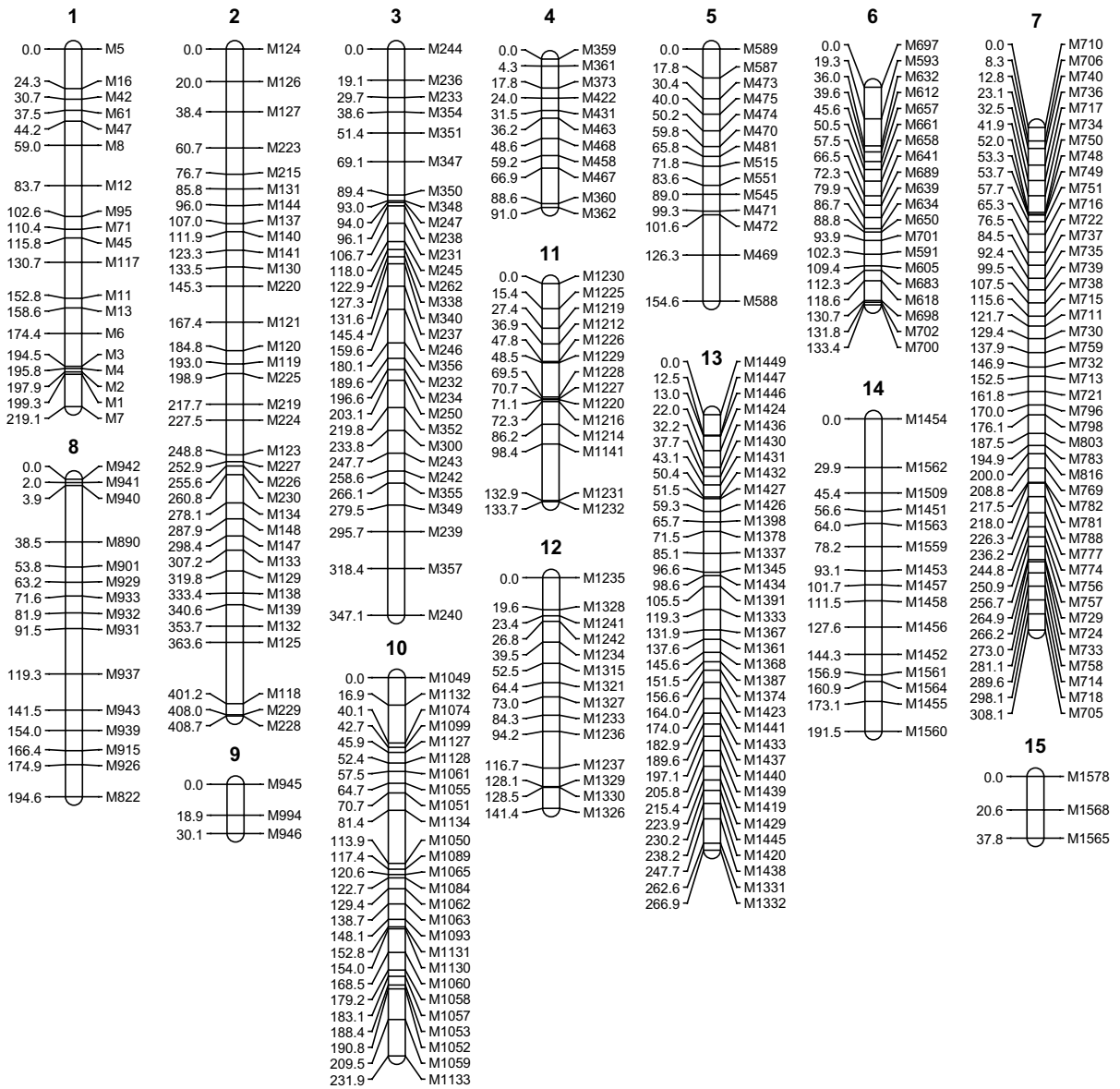
Among the 296 markers used to construct the linkage map, 268 (90.54%) exhibited significant distortion from the expected Mendelian segregation ratio (Table 1). The number of distorted markers was found at a high density on all LGs (Table 1). Most of the distorted markers on all LGs favored heterozygous genotypes.

### Identification of QTLs for salt tolerance

The results of the QTL analysis for salt tolerance were summarized in Table 2. Two QTL, designated *qSaltol\_3.1* and *qSaltol\_7.1*, were detected for the AUDPC calculated from the LWS for salt tolerance. The *qSaltol\_3.1* was mapped on LG3 and accounted for 26.71% of the tolerance score variation in the population. It showed an additive effect of -1.58 and a dominant effect of 2.81. The *qSaltol\_7.1* was mapped on LG7 and accounted for 11.12% of the tolerance score variation in the population. It showed an additive effect of -0.07 and a dominant effect of -2.85. Similarly, two QTLs, *qSaltol\_3.1* and *qSaltol\_7.1*, were detected for the AUDPC calculated from the PSP. The *qSaltol\_3.1* accounted for 22.55% of the survival variation in the population and showed an additive effect of 95.33 and a dominant effect of -59.42, while the *qSaltol\_7.1* explained 14.90% of the trait variation in the population and showed additive and dominant effects of 8.73 and 126.11, respectively.

**Table 1** Distribution of SNP markers and average distance between markers on 15 linkage groups of zombi pea BC<sub>1</sub>F<sub>2</sub> population derived from the cross TVNu240 × (TVNu240 × S261)

Linkage group	Number of SNP markers	Number of distorted SNPs	Map length (cM)	Average marker spacing (cM)
LG1	19	18	219.13	11.53
LG2	34	26	408.74	12.02
LG3	30	30	347.11	11.57
LG4	11	9	90.97	8.27
LG5	14	14	154.61	11.04
LG6	20	18	133.43	6.67
LG7	43	31	308.09	7.16
LG8	15	14	194.56	12.97
LG9	3	3	30.06	10.02
LG10	26	26	231.86	8.92
LG11	14	14	133.65	9.55
LG12	14	13	141.42	10.1
LG13	35	34	266.92	7.63
LG14	15	15	191.51	12.77
LG15	3	3	37.85	12.62
Average	19.73	17.87	192.66	9.76
Total	296.00	268.00	2,889.90	-



**Fig. 3** A SNP-based genetic linkage map of the zombi pea BC<sub>1</sub>F<sub>2</sub> population derived from the cross TVNu240 × (TVNu240 × S261)

**Table 2** TLs detected for salt tolerance in zombi pea BC<sub>1</sub>F<sub>2</sub> population of the cross TVNu 240 × (TVNu 240 × S261)

Trait	QTL name	LG	Position (cM)	Left marker	Right marker	LOD score	PVE (%)	Additive effect	Dominant effect
ADUPC leaf wilt score	<i>qSoltol_3.1</i>	3	118.0	M231	M245	7.01	26.71	-1.58	2.82
	<i>qSoltol_7.1</i>	7	32.6	M717	M734	3.21	11.12	-0.07	-2.85
ADUPC plant survival	<i>qSoltol_3.1</i>	3	116.4	M231	M245	5.69	22.55	95.33	-59.42
	<i>qSoltol_7.1</i>	7	32.6	M717	M734	4.40	14.90	8.73	126.11

## Discussion

Zombi pea is an underutilized crop with several varieties/forms of wild progenitor. Wild zombi pea shows wide distribution, and several accessions of wild zombi pea have been reported to be resistant to biotic and abiotic stresses. The wild zombi pea can be used as a gene donor for the breeding of cowpeas, an important legume crop. This study identifies two QTLs controlling salt tolerance in wild zombi pea accession S261 (AusTRCF 322105). The number of QTLs for salt tolerance detected in our study was similar to that previously detected in wild zombi pea accession JP235908, where three QTLs were detected (Dachapak et al. 2019). However, the QTLs controlling the resistance in these two wild zombi pea accessions are likely to be different. Dachapak et al. (2019) showed that *qSaltol.1* was the major QTL controlling salt tolerance, and, based on comparative map analysis, the candidate gene for this QTL was *Vigan.01G480200.01*, which encodes plasma membrane H<sup>+</sup>-ATPase. In the zombi pea reference genome (Naito et al. 2022), *Vigan.01G480200.01* is equivalent to *Vigve.0013s057500.01*, located on the scaffold scf0013. In our study, *qSaltol\_3.1*, which is the major QTL controlling salt tolerance, was mapped between markers M231 and M245. These markers are located in nearly the same position on the scaffold scf0024 of the zombi pea reference genome (Naito et al. 2022; <https://viggs.dna.affrc.go.jp>). We explored a 500-kb region (scf0024:389,500...889,499) covering markers M231 and M245 (about 250 kb away from M231 and 250 kb away from M245). There were 16 annotated genes in the 500-kb regions, of which 10 encode uncharacterized proteins, 1 encodes hypothetical proteins, and 5 encode proteins with known functions (Supplementary Table S1). Among the genes with known function, *Vigve.0024s001200.01* encoding methionine sulfoxide reductase (MSR) and *Vigve.0024s002000.01* encoding protein-L-isoaspartate O-methyltransferase (PIMT) are of interest. MSRs are thioredoxin-linked enzymes involved in the conversion of methionine sulfoxide to methionine and have been reported to play roles in various stress tolerances (Romero et al. 2004; Kwon et al. 2007; Oh et al. 2010; Li et al. 2012; Tarrago et al. 2012; Laugier et al. 2013; Lee et al. 2014; Roy and Nandi 2016). In a recent study, Cai et al. (2023) demonstrated that methionine sulfoxide reductase B5

(MSRB5) from *Arabidopsis thaliana* L. functions as a salt-stress protector in both *A. thaliana* and rice (*Oryza sativa* L.) via modulation of Na<sup>+</sup>/K<sup>+</sup> homeostasis. PIMT is a protein-repairing enzyme that repairs isoaspartyl residues in proteins and re-establishes protein structure and function. PIMT repairs isoaspartyl residues by catalyzing the conversion of atypical isoaspartyl residues to normal aspartyl residues via the S-adenosyl methionine-dependent methylesterification reaction (Lowenson and Clarke 1992; Aswad et al. 2000). Studies in *A. thaliana* showed that PIMT is involved in plant growth and survival under salt stress by increasing the efficiency of ROS-scavenging enzymes (Ghosh et al. 2020a), repairing isoaspartyl damage to antioxidant enzymes, and increasing heat and oxidative stress tolerance (Ghosh et al. 2020b). Thus, *Vigve.0024s001200.01* and *Vigve.0024s002000.01* can be considered as the candidate genes for salt tolerance at *qSaltol\_3.1*. Notably, *Vigve.0024s002000.01* is closer to the markers M231 and M245 than *Vigve.0024s001200.01*.

In the case of *qSaltol\_7.1*, this QTL was located between markers M717 and M734. These markers were located on the scaffold scf0049 at positions 183,906 and 607,757 of the zombi pea reference genome (Naito et al. 2022; <https://viggs.dna.affrc.go.jp>), with a distance of 423.85 kb between them. There were 46 genes in this region (Supplementary Table S2). Among these genes, *Vigve.0049s001800.01* encoding 3-ketoacyl-CoA synthase (KCS), *Vigve.0049s001900.01* encoding L-type lectin-domain containing receptor kinase VII.1 (LecRK-VII.1), *Vigve.0049s002200.01* encoding lipoxygenase 3 (LOX3), *Vigve.0049s004000.01* encoding CBS domain-containing protein 5 (CBSX5), *Vigve.0049s005500.01* encoding NAC25, and *Vigve.0049s004100.01*, *Vigve.0049s004200.01*, and *Vigve.0049s005100.01* encoding asparagine synthetase (ASNS) are of interest. KCSs regulate the synthesis of very long-chain fatty acids that play important roles in the survival and development of plants. Overexpression of the KCS gene from *Vitis vinifera* L. in *A. thaliana* resulted in increased tolerance to salt stress during the germination and seedling stages (Yang et al. 2020). LecRKs are proteins with an extracellular lectin motif and an intracellular kinase domain. The *LecRK-VII.1* gene has been shown to be involved in the salt-stress response in *A. thaliana* (Zhang et al. 2017). LOXs catalyze the



oxidation of polyunsaturated fatty acids into fatty acid hydroperoxides and play important roles in defense responses against biotic and abiotic stresses (Singh et al. 2022). A recent study in apple (*Malus domestica* L.) showed that expression of *LOX3* is induced by salt stress, overexpression of *LOX3* in apple calli improved salt tolerance, and heterotopic expression of *LOX3* in *A. thaliana* enhanced salt stress tolerance by enhancing the ability to scavenge reactive oxygen species (ROS) (Chen et al. 2022). Studies on CBSX proteins in plants were only recently initiated. CBSXs directly regulate the activation of thioredoxins, thereby controlling cellular H<sub>2</sub>O<sub>2</sub> levels and modulating both plant development and growth. Studies in *A. thaliana*, tobacco (*Nicotiana tabacum* L.), and rice found that CBSXs were related to abiotic stresses such as salinity, heavy metals, and oxidative stress. Increased expression of *CBSX5* is associated with response to salt stress in *A. thaliana* (Kushwaha et al. 2009), rice (Kushwaha et al. 2009), mungbean (*Vigna radiata* (L.) R. Wilczek) (Xu et al. 2023), and quinoa (*Chenopodium quinoa* Willd.) (Hosseini et al. 2023). NAC transcription factors play crucial roles in plant development and stress responses. Arabidopsis plants overexpressing the *NAC25* gene from *Malus baccata* (L.) Borkh showed increased tolerance to salt stress via enhanced scavenging capability of ROS (Han et al. 2020). Upregulation of an *ASNS* gene in rice (*Oryza sativa* L.) enhanced salt tolerance under salt stress conditions (Deng et al. 2022). Thus, *Vigve.0049s001800.01*, *Vigve.0049s001900.01*, *Vigve.0049s004000.01*, *Vigve.0049s004100.01*, *Vigve.0049s004200.01*, and *Vigve.0049s005100.01* can be considered candidate genes for salt tolerance at *qSaltol\_7.1*. Further research is necessary to confirm the association between all the candidate genes and salt tolerance in zombi pea. Nonetheless, the QTLs and candidate genes controlling salt tolerance in S261 and JP235908 appeared to be different. Both the accessions S261 and JP235908 belong to the variety *ovata* of *V. vexillata*. Pienaar and Kok (1991) proposed that the variety *ovata* is native to southern Africa. However, unlike the accession JP235908 that is collected from a mountainous region relatively far from a coastal region of South Africa ([https://www.gene.affrc.go.jp/databases-plant\\_search\\_en.php](https://www.gene.affrc.go.jp/databases-plant_search_en.php)), the accession S261 is collected near a coastal region of Australia (<https://www.genesys-pgr.org/a/6204fdaa-fe0c-4f8d-a12f-a33c159e0968>). The contrasting

origins and ecologies where the two accessions evolved may explain the difference in QTLs controlling salt tolerance. Nonetheless, pyramiding QTLs/genes from these two accessions may provide high tolerance to salt stress.

**Author contributions** KL, PS and LW conceived and designed the study. KL and KA conducted all the experiments. KL analyzed all the data. KL, PS and LW secured research funds. KL wrote the manuscript. PS edited the manuscript. KL and PS revised the manuscript. All authors reviewed the manuscript.

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**Data availability** The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Declarations**

**Conflict of interest** The authors declare that they have no conflict of interest.

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