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Molecular loci associated with seed isoflavone content may underlie resistance to soybean pod borer (*Leguminivora glycinivorella*)

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Abstract

Soybean pod borer (SPB) (*Leguminivora glycinivorella* (Mats.) Obraztsov) causes severe loss of soybean (*Glycine max* L. Merr.) seed yield and quality in some regions of the world, especially in north-eastern China, Japan and Russia. Isoflavones in soybean seed play a crucial role in plant resistance to diseases and pests. The aim of this study was to find whether SPB resistance QTL are associated with soybean seed isoflavone content. A cross was made between 'Zhongdou 27' (higher isoflavone content) and 'Jiunong 20' (lower isoflavone content). One hundred and twelve $F_{5:10}$ recombinant inbred lines were derived through single-seed descent. A plastic-net cabinet was used to cover the plants in early August, and thirty SPB moths per square metre were put in to infest the soybean green pods. The results indicated that the percentage of seeds damaged by SPB was positively correlated with glycitein content (GC), whereas it was negatively correlated with genistein (GT), daidzein (DZ) and total isoflavone content (TI). Four QTL underlying SPB damage to seeds were identified and the phenotypic variation for SPB resistance explained by the four QTL ranged from 2% to 14% on chromosomes Gm7, 10, 13 and 17. Moreover, eleven QTL underlying isoflavone content were identified, and ten of them were encompassed within the same four marker intervals as the SPB QTL (BARC-Satt208-Sat292, Satt144-Sat074, Satt540-Sat244 and Satt345-Satt592). These QTL could be useful in marker-assisted selection for breeding soybean cultivars with both SPB resistance and high seed isoflavone content.

Key words: soybean — pod borer — isoflavones — QTL (Quantitative Trait Locus)

Soybean pod borer (*Leguminivora glycinivorella* (Mats.) Obraztsov) is a severe pest in north-eastern China, Japan, Russia and Indonesia (Talekar 1987, Sharma 1998). SPB feeds on young soybean (*Glycine max* L. Merr.) seeds, causing yield loss, seed quality reduction and sale price decreases (Edmonds et al. 2000). SPB injury occurs during the pod-filling stage. Large numbers of eggs are laid on immature pods by *L. glycinivorella* moths. The emerging larvae feed on green soybean seed (Hsu et al. 1965, Turnipseed and Kogan 1976). Currently, field control of SPB relies mainly on chemical pesticides (Zhao 2004). Pesticides are costly, pollute the environment and kill non-target organisms (Yan et al. 1997).

Cultivation of resistant cultivars is an effective method to control SPB. The development of insect-resistant soybean varieties could reduce pesticide usage in controlling insects, but little success has been achieved so far. Some studies indicate that insect resistance is quantitatively inherited (Sharma 2008, Jun et al. 2013). Therefore, selection for soybean cultivars with high resistance against SPB requires the evaluation of resistance in multiple environments over several years. In traditional breeding programme, that is time consuming, labour intensive and inefficient. A molecular marker technique (marker-assistant selection, MAS) has emerged as a useful tool for the selection of many complex traits including disease and insect resistance. MAS can reduce costs and time to cultivar release, and thereby increase the efficiency of breeding programmes (Zhao et al. 1994, 2008, Huang et al. 2000). However, little attention has been paid to SPB resistance breeding by MAS (Zhao et al. 2008).

Dixon (1999) concluded that isoflavones were particularly prevalent in the *Papilonoideae* subfamily of the *Leguminosae*, in which they were widely distributed and functioned as anti-insect compounds. Soybean seed contains about 1000–3000 μg/g of isoflavone based on seed mass. Isoflavones, 3-phenyl-4H-1-benzopyran-4-ones, are phenolic secondary metabolites produced uniquely in legumes with many special biologic functions (Wang and Murphy 1994, Singh et al. 2003). In soybean, four chemical forms of isoflavones (aglycone, glucoside, acetylglucoside, malonylglucoside) are found and each consists of three different types, of which the aglycones are comprised of daidzein (DZ), genistein (GT) and glycitein (GC). Their glucosides are named daidzin, genistin and glycitin. The acetylglucosides include 6″-O-acetyldaidzin, 6″-O-acetylgenistin and 6″-O-acetylglycitin. While the malonylglucosides contain 6″-O-malonyldaidzin, 6″-Omalonylgenistin, and 6″-O-malonylglycitin. Isoflavones have insect feeding-deterrent activities (Sutherland et al. 1980, Lane et al. 1987) and play vital defensive roles against many pests, such as stinkbugs (*Nezara viridula*; Piubelli et al. 2003), *Anticarsia gemmatalis* (Giorla et al. 2005), soybean cyst nematodes (*Heterodera glycines*; Afzal et al. 2009) and aphids (*Aphis glycines*; Meng et al. 2011a). In chickpeas (*Cicer arietinum*), two components of their isoflavonoids, maackiain and judaicin play important roles in decreasing the susceptibility to the pod borer *Helicoverpa armigera* (Simmonds and Stevenson 2001). Maackiain is the only isoflavone to be active against *Spodoptera frugiperda*, and judaicin is the only isoflavone to be active against *S. littoralis* (Simmonds 2003). Significant differences in isoflavone contents have been observed between insect resistant and susceptible soybean cultivars. The insect-resistant cultivars contain significantly more phytoalexins than most susceptible ones. As glyceollin is often the best resistance induction indicator, so the concentration of phytoalexin in soybean seedlings could be used to identify insect-resistant germplasm in breeding programmes (Liu et al. 1992). In a study about the association between glyceollin concentration and soybean root resistance to a root knot nematode (*Meloidogyne incognita*), the resistant cultivars showed significant glyceollin

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accumulation two to three days after inoculation, while there was no accumulation of glyceollin in susceptible variety (Kaplan et al. 1980, Veech 1982). In addition, several isoflavones (such as judaicin, judaicin 7-Oglucoside and judaicin 7-O-6″-O-malonylglucoside) in the wild relatives of chickpea and pterocarpans (maackiain 3-O-glucoside and maackiain 3-O-6″-O-malonyl glucoside) have been reported to act against *H. armigera* by antibiosis (Stevenson and Veitch 1996, Sharma et al. 2005). Although isoflavones have been reported to correlate with resistance to pests and diseases, few studies have addressed their effects on SPB resistance by 2014.

QTL underlying isoflavone content was first reported in the mapping population 'Essex' × 'Forrest' (Njiti et al. 1999). Subsequently, numbers of QTL associated with isoflavone contents of soybean were reported (Meksem et al. 2001, Kassem et al. 2004, Zeng et al. 2009, Yang et al. 2011, Akond et al. 2014). Two genes, *IFS1* and *IFS2*, were reported to associate with isoflavone concentration in soybean seeds (Cheng et al. 2008), and twenty-six more candidate genes that might be involved in isoflavones accumulation in soybean seed were identified (Akond et al. 2013).

However, few QTL underlying SPB resistance were identified by 2014 (Zhao 2004, Zhao et al. 2008). The objectives of this study were to identify QTL for SPB resistance, to identify QTL associated with seed isoflavone content and to facilitate genetic improvement of soybean to combine both SPB resistance and high isoflavone content by MAS.

Materials and Methods

Plant material: One hundred and twelve $F_{5:10}$ recombinant inbreed lines (RILs) were advanced from a cross between 'Zhongdou 27' (high individual and total isoflavone contents in seed, developed by the Chinese Academy of Agriculture, Beijing, China) and 'Jiunong 20' (low individual and TI contents in seed, developed by Jinlin Academy of Agriculture, Jilin, China).

Field experiment: The seeds of the recombinant inbred lines were increased in fields without SPB infestation, so that unintentional selection could be minimized. One hundred and twelve RILs, two parents and several check varieties were planted on May 5 of 2009 at the Experimental Station of Northeast Agricultural University, Harbin, China (45°N, fine-mesic chernozen soil), utilizing a randomized complete block design with three replications, with rows 3 m long, 0.70 m apart and with a space of 6 cm between two plants. When setting pods, all the plants were covered by a plastic-net cabinet to control the insect infestations. Then the plants were infested by SPB with a density of thirty SPB moths per square metre (several times high than natural field conditions) when soybean pods were first developing. The higher insect infestation density ensured significant damage could be scored. Similar methods were used with corn earworm (*H. armigera*) in maize (Rector et al. 1998, 2000) and with SPB in soybean (Zhao et al. 2008). The moths were trapped with a sweep net when they had just emerged in several neighbouring soybean fields. Twenty non-infested plants were selected from each genotype to act as seed donors that were later used to replicate the assays of resistance to SPB. The percentage of damaged seeds (PDS) among infested plants was scored after harvest (PDS = (number of seeds damaged by SPB/total number of seeds) × 100; Zhao et al. 2008). The means of the individual and total seed isoflavone contents were calculated using data from the one hundred and twelve lines in present study. In addition, data from the seven environments used in Zeng et al. (2009) were included.

Phenotypic data analyses: The frequency distribution and statistic parameters for RILs were analysed using the SAS procedure (PROC.COR.SAS; SAS Institute, Cary, NC, USA).

Isoflavone extraction and analyses: Isoflavone concentrations were determined using HPLC as described by Vyn et al. (2002). One hundred grams of soybean flour milled with a cyclone mill (Perten Laboratory Mill 3100, speed 1100 *g*, Perten Instruments, Hägersten, Sweden) was used for each extraction with 10 ml of 80% (v/v) ethanol in 10-ml Falcon™ tube that was slowly vortexed for 1 h and then left overnight. The extraction was hydrolysed by 2 ml HCl (35% v/v) to return the derivatives to DZ, GC and GT. The extraction was filtered through PTFE 0.45 μm membrane (Sartorius, Göttingen, Germany), and 10 μl of the filtrate was used to detect the isoflavone content by ASI-100 HPLC (Dionex, St Louis, MO, USA). The following conditions for HPLC were employed: solvent A was double-distilled water (ddH_2O); and solvent B was methanol (chromatography purity); solvent A : B ratio was 50 : 50; the solvent flow rate was 1.0 ml/min; and the temperature of column was kept at 50°C; the Dionex UV-170 detector monitored at 254 nm and the UV spectra were recorded. The area responses were integrated by software Dionex 2.0. The quantification of isoflavones was determined by reference to an external standard.

Molecular linkage map construction: Total DNA of parents and each RIL were isolated from freeze-dried leaf tissues by the CTAB method as described by Doyle (1990). SSR analysis was performed with the BARC-SSR primers developed by Cregan et al. (1999). Mapmaker/EXP version 3.0 b (Lander et al. 1987) was used for genetic linkage analyses. The genetic linkage map was drawn using Mapchart 2.1 (Voorrips 2002).

QTL analyses: QTL mapping for each trait was performed based on the separate phenotypic values using composite interval mapping (CIM) module (Zeng 1993, 1994) with QTL Cartographer Version 2.5 (Wang et al. 2006). The threshold of LOD score for evaluating the statistical significance of QTL effects was determined by 1000 permutations using the Zmapqtl program in QTL Cartographer (Churchill and Doerge 1994).

A LOD value corresponding to an experiment-wise threshold of a = 0.05 was used to declare a QTL as significant. The estimate of the QTL position was the point of maximum LOD score in the region under consideration.

Results

Phenotypic analyses of damaged seeds by SPB

The percentage of damaged seeds (PDS) of the two parents was 7.56% for 'Zhongdou 27' and 16.96% for 'Jiunong 20' and represented a significant difference in SPB resistance (Table 1). The ranges of PDS among one hundred and twelve RILs from the cross of 'Zhongdou 27' × 'Jiunong 20' were from 3.19% to 21.54% (Table 1) and displayed a continuous frequency distribution (Fig. 1). The RILs showed a low variation coefficient value (<0.5). Both skewness and kurtosis values of PDS were <1.0, suggesting that the segregation of this trait fits a normal distribution model (Table 1).

Phenotypic analyses of isoflavone content

The distribution of isoflavone components scored for the environments varied (Fig. 1). They ranged from 509.16 μg/g to 2416.31 μg/g for DZ, from 116.81 μg/g to 405.13 μg/g for GC, from 397.85 μg/g to 2631.25 μg/g for GT and from 1149.85 μg/g to 4438.25 μg/g for total isoflavones (TI). Mean DZ, GC, GT and TI contents for 'Zhongdou 27' were 1934.41 μg/g, 305.15 μg/g, 1930.36 μg/g and 3821.09 μg/g, respectively. While mean DZ, GC, GT and TI contents for 'Jiunong 20' were 1456.09 μg/g, 181.92 μg/g, 1109.57 μg/g and 2042.38 μg/g, respectively. The mean DZ, GC, GT and TI for 'Zhongdou 27' (with high SPB resistance) were higher than those of 'Jiunong 20' (with low SPB resistance), suggesting the existence of some relation between isoflavone contents and SPB resistance. Many

Table 1: Statistic analyses of the percentage of damaged seeds for parents and RILs population

	Parents		RIL population				
Trait	Zhongdou 27'	'Jiunong 20'	Mean	Range	SE[1]	Skewness	Kurtosis
PDS[2]	7.56	16.96	9.81	3.19–21.54	21.49	0.96	0.11

[1]Standard variation; [2]percentage of damaged seeds caused by pod borer.

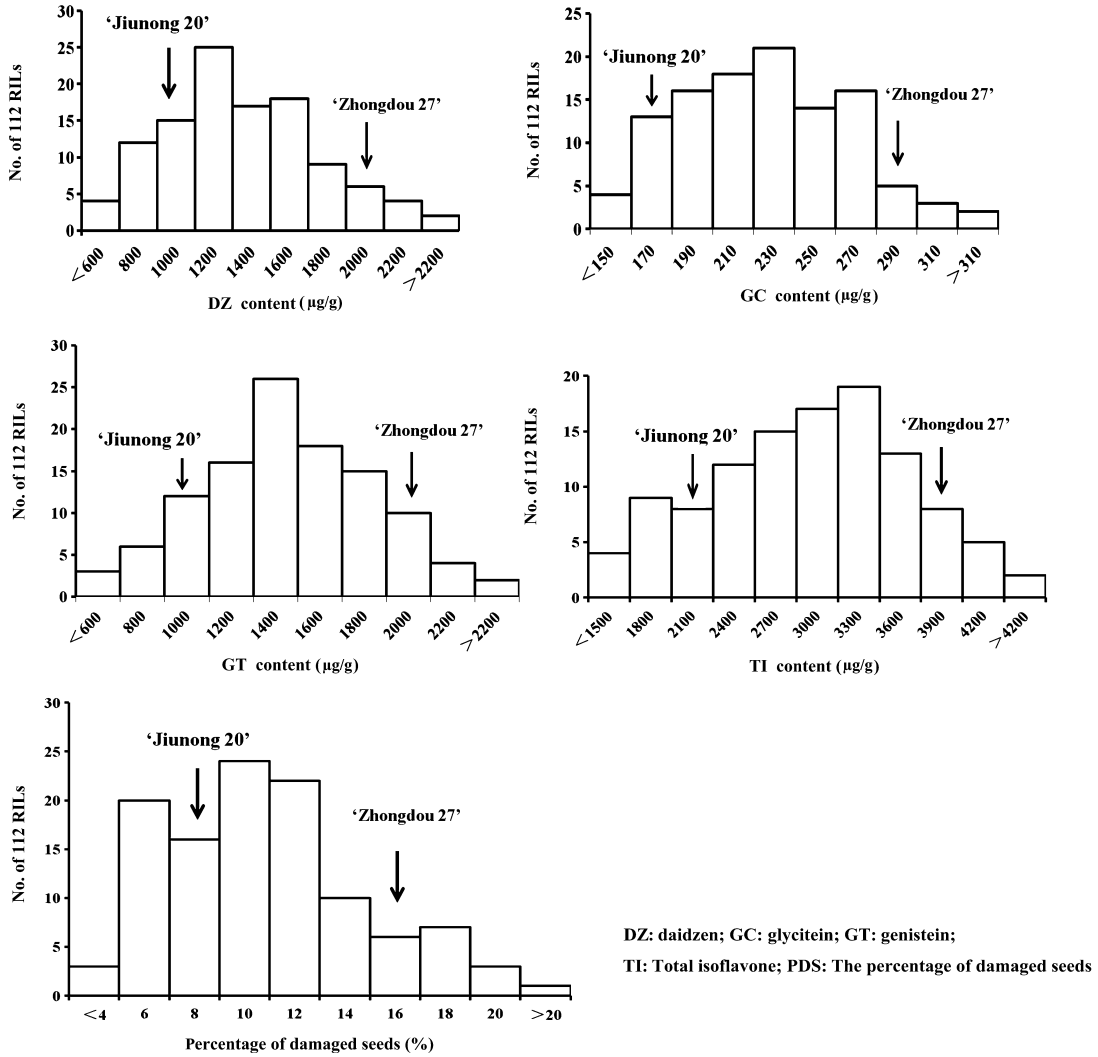

Fig. 1: Frequency distribution of PDS and DZ, GC, GT and TI content among the 112 RILs from cross of cultivar 'Zhongdou 27' × 'Jiunong 20'. Note: Arrows indicate the location of means of parents. DZ: daidzein; GC: genistein; TI: total isoflavone

individuals showed transgressive segregation compared to the parents of the population (Fig. 1).

Correlation between SPB resistance and isoflavone

A significantly positive correlation existed between percentage of damaged seeds by SPB and GC isoflavone content, and a significantly negative correlation existed between PDS by SPB and DZ, GT and TI isoflavone contents (Table 2).

Identification of QTL associated with SPB resistance

Four QTL underlying SPB resistance were detected (Table 3). The QSPBD2_1 was located on LG D2 (Gm 17) and flanked by Satt208 and Sat_292, the QSPBF_1 was located on LG F (Gm 13) and flanked by Satt144 and Sat_074. The QSPBM_1 was identified on LG M (Gm 7) between Satt540 and Sat_244, and the QSPBO_1 was detected on LG O (Gm10) between Satt345 and Satt592. The phenotypic variation explained by the four QTL ranged from 2% to 14%. The beneficial alleles of all four QTL were contributed by 'Zhongdou 27' (the high isoflavone variety).

Identification of QTL associated with isoflavone content

Eleven QTL underlying isoflavone content were detected (Table 4). Three QTL, QDZD2_1 and QDZ D2_2 located on D2 (Gm 17) and QDZF_1 located on LG F (Gm 13), appeared to

Table 2: Correlation between the percentages of damaged seeds caused by pod borer and the content of isoflavone

Trait	DZ	GC	GT	TI
PDS	−0.1316[1]	0.2584[2]	−0.5019[2]	−0.3588[2]

DZ, daidzein; GC, glycitein; GT, genistein; TI, total isoflavone; PDS, the percentage of damaged seeds; [1]significant at 0.05 level; [2]significant at 0.01 level.

Table 3: QTL associated with SPB resistance

QTL	LG (Gm)	Marker interval	LOD[1]	R^2(%)[2]
QSPBD2_1	D2 (17)	Satt208-Sat_292	2.30	1.97
QSPBF_1	F (13)	Satt144-Sat_074	27.00	11.47
QSPBM_1	M (7)	Satt540-Sat_244	3.92	14.00
QSPBO_1	O (10)	Satt345-Satt592	2.01	6.54

[1]LOD is log of odd score; [2]R^2 is R-square or the proportion of the phenotypic data explained by the marker locus.

underlie seed DZ content. Two QTL, QGTF_1 located on LG F (Gm 13) and QGTM_1 located on LG M (Gm 7), underlay seed GT content. Two QTL, QDZO_1 and QGCM_1 located on LG O (Gm10) and LG M (Gm 7), underlay seed GC content. Four QTL, QDZD2_1 located on D2 (Gm 17), QTIF_1 located on LG F (Gm 13), QTIO_1 located on LG O (Gm10) and QTIM_1 located on LG M (Gm 7), appeared to underlie TI. The phenotypic variation explained by these QTL ranged from 3.3% to 11.8%.

Comparison of QTL underlying the resistance to SPB and isoflavone content

Here, four marker intervals (Satt208-Sat_292 in D2 (Gm 17), Satt144-Sat_074 in F (Gm 13), Satt540-Sat_244 in M (Gm 7) and Satt345-Satt592 in O (Gm 10)) were all inferred to underlie both SPB resistance and isoflavone contents in soybean seed. That strongly suggested that SPB resistance was related to isoflavone content. Moreover, the QTL associated with SPB resistance identified in this study were compared with the QTL underlying individual and total isoflavone contents detected in a previous study (Zeng et al. 2009, Fig. 2).

The QTL QSPBD2_1 associated with SPB resistance located on LG D2 (Gm17) (Satt208-Sat_292; Fig. 2, Table 3), while in the same marker interval, QTID2_1 was previously detected to underlie TI content. QTL QSPBF_1 underlying SPB resistance located on LG F (Gm13) between Satt144-Sat_074 (Fig. 2, Table 3), while QDZF_1, QGTF_1 and QTIF_1 for DZ, GT and TI contents, respectively, were previously detected in the same marker interval (Zeng et al. 2009). QTL QSPBM_1 associated with SPB resistance located on LG M marked by Satt540-Sat_244. Further, QTL QGCM_1, QGTM_1 and QTIM_1 responsible for GC, GT and TI content, respectively, were also flanked by marker Satt540. QTL QSPBO_1 associated with SPB resistance located on LG O (Gm 10) was flanked by Satt345 and Satt592, while QTL QGCO_1 associated with GC content was linked to marker Satt592 but were between markers Satt592 and Satt533. The above results suggested that the genes controlling SPB resistance and seed isoflavone content were located in the same genomic regions, or in some case might be underlain by the same genes.

Table 4: QTL associated with isoflavones

Trait	QTL	LG	Marker	LOD[1]	R^2 (%)[2]
DZ	QDZD2_1	D2	Satt208-Sat_292	2.33	5.57
	QDZD2_2	F	Satt255-Satt528	2.11	4.87
	QDZF_1	F	Satt144-Sat_074	3.80	8.32
GT	QGTF_1	F	Satt144-Sat_074	5.27	6.63
	QGTM_1	M	Satt540-Sat_244	2.56	11.76
GC	QDZO_1	D2	Satt345-Satt592	3.18	4.40
	QGCM_1	M	Satt540-Sat_244	6.20	5.59
TI	QDZD2_1	D2	Satt208-Sat_292	5.71	3.31
	QTIF_1	F	Satt144-Sat_074	4.44	10.88
	QTIO_1	O	Satt345-Satt592	2.00	7.91
	QTIM_1	M	Satt540-Sat_244	2.06	5.33

[1]LOD is log of odd score; [2]R^2 is R-square or the proportion of the phenotypic data explained by the marker locus.

Discussion

Soybean pod borer (*L. glycinivorella*) has long been known as the most destructive soybean pest in north-eastern China, Japan and Russia (Zhao et al. 2008). Some soybean cultivars with partial resistance to SPB had been released through selection of morphological and biological traits correlated to SPB resistance (Zhao et al. 1994). However, the released soybean varieties have not been used effectively because of a limited resistance and lower yield caused by linkage drag. Also, the progress in developing soybean varieties with pest resistance has been slow because of the lack of information on the mechanisms of insect resistance, the genes involved and the nature of gene action, especially the lack of an effective selection method, such as marker-assistant selection (Sharma 2008).

Isoflavones were reported to be correlated with insect resistance, including *H. armigera*, *S. frugiperda* and *S. littoralis* in chickpea (Simmonds 2003). However, there is no information to indicate whether isoflavone compounds would influence other pests such as soybean pod borer. Soybean is rich for isoflavone in seed. Genistein and daidzein are the two most important isoflavones presented in soybean seeds, representing 64% and 23% of total isoflavones, respectively (Naim et al. 1974, Carrao-panizzi and Kitamura 1995). Some studies had showed that these isoflavones are the most effective compounds in antixenotic and antibiotic relations (Fisher et al. 1990, Graham and Graham 1991). Here, the correlations between PDS caused by SPB and four isoflavones (GC, DZ, GT, TI), showed that SPB resistance was significantly positively correlated with DZ, GT and TI contents (Table 2). Along with the increase of DZ, GT and TI, SPB fed seeds were constantly reduced, which indicated that soybean cultivars, containing high contents of DZ, GT and TI, had potential resistance to SPB. Similar results were found in other studies. For example, the content of DZ, GT and TI in soybean leaves was positively correlated with the resistance to soybean aphid (Meng et al. 2011b). Piubelli et al. (2003) found that the increased production of isoflavone was correlated with the resistance to *Nezara viridula*. Giorla et al. (2005) reported that feeding soybean with higher content of GT to *Anticarsia gemmatalis* led to high mortality and reduced the weight of larvae and pupa. Isoflavones were also been observed to correlate with plant resistance against pathogens. Lozovaya et al. (2004) reported the function of soybean isoflavones in plant resistance to soybean sudden death syndrome (SDS) and found that the DZ content in soybean hairy roots of resistant variety (PI567.374) was higher than that of susceptible cultivar (Spencer).

Four QTL (QSPBD2_1, QSPBF_1, QSPBM_1 and QSPBO_1) were identified to associate with SPB resistance in

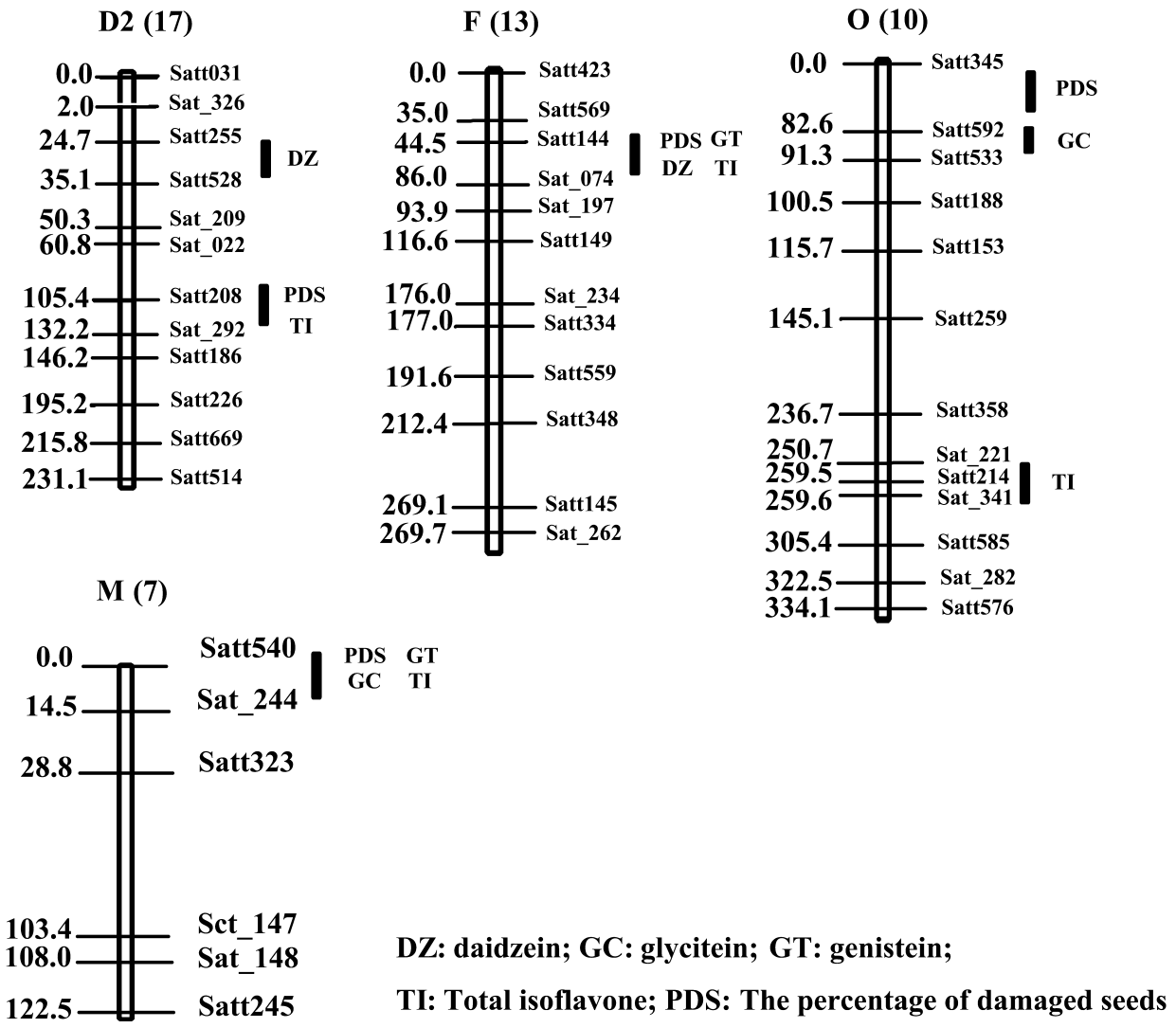

Fig. 2: Comparison of QTL for both SPB resistance and average isoflavone content identified in this study and previous work (Zeng et al. 2009). Note: QTL are indicated by a bar on the right of linkage group. The markers used were developed by Perry–Cregan's research group

the present study. After the comparison with the nine QTL associated with individual and total isoflavone contents that were detected in previous work (Zeng et al. 2009) using the same RIL population (Fig. 2), there was agreement that QTL QSPBD2_1 for SPB resistance and QTID2_1 underlying TI content were both located on LG D2 (Gm17) and mapped between Satt208 and Sat_292 (Fig. 2). That suggested that environmentally stable genes controlling both SPB resistance and TI content existed in this genetic region. QTL for the resistance against *Prodenia litura* (L.) Fabricius and *Bemisia tabaci* (Gennadius) were also reported to be anchored on LG D2 (Gm17; Wang et al. 2004), indicating more genes that regulated pest resistance are located on chromosome 17.

QTL QSPBF_1 associated with SPB resistance and three QTL (QDZF_1, QGTF_1 and QTIF_1) for DZ, GT and TI content (Zeng et al. 2009) were all confirmed to be located on LG F (Gm13) between markers Satt144 and Sat_074 (Fig. 2). As the PDS caused by SPB was negatively correlated with DZ, GT, and TI content, this genetic region might contain the genes underlying both SPB resistance and isoflavone accumulation. An aphid resistance locus was detected near the same marker Satt144 by Meng et al. (2011a). Moreover, QTL for corn earworm resistance was also reported on the same chromosome (Rector et al. 1998, 1999, 2000). However, the relationship between genes underlying isoflavone individuals and those resistant to pests should be probed by fine mapping, mutation and transgenic analyses.

QTL QSPBM_1 underlying SPB resistance was located on LG M (Gm 7) between marker Satt540 and Sat_244 (Fig. 2), while QGCM_1, QGTM_1 and QTIM_1 responsible for isoflavone GC, GT and TI content were linked to the same SSR marker, Satt540 on LG M (Gm7) (Fig. 2). An isoflavone QTL near Satt540 was detected by Primomo et al. (2006) using 'AC756' × 'RCAT Angora' RILs in two locations of Canada. In other studies, Satt540 was verified to be associated with resistance to corn earworm (Rector et al. 1998, 1999, 2000), common cutworm (Komatsu et al. 2005), aphids (Li et al. 2007) and white mould (Guo et al. 2008). Therefore, loci underlying both pest/disease resistance and isoflavone accumulation existed near Satt540. The genes on chromosome 7 near Satt540 may be clustered or pleiotropic. Therefore, Satt540 will be a valuable molecular marker to facilitate soybean breeding with enhanced pest/disease resistance and enriched seed isoflavones by marker-assistant selection.

Here, the QSPBO_1 associated with SPB resistance was detected on LG O (Gm10) between Satt345 and Satt592. QGCO_1 associated with isoflavone GC content was also close to the same marker Satt592. Moreover, Satt592 associated with resistance to soybean cyst nematode (SCN) as a diagnostic marker (Arelli et al. 2010). QTL underlying resistances to southern root knot nematode (Tamulonis et al. 1997, Li et al. 2001), to *Prodenia litura*, (Fabricius) (Fu et al. 2007) and to *Megacota cribraria* (Fabricius) (Xing et al. 2008) were also found on the same chromosome (Gm 10).

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Conflict of interests

The authors declare that they have no conflict of interests in the research.

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