A multiomics approach reveals the pivotal role of subcellular reallocation in determining rapeseed resistance to cadmium toxicity

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Abstract

Oilseed rape (Brassica napus) has great potential for phytoremediation of cadmium (Cd)-polluted soils due to its large plant biomass production and strong metal accumulation. Enhanced plant Cd resistance (PCR) is a crucial prerequisite for phytoremediation through hyper-accumulation of excess Cd. However, the complexity of the allotetraploid genome of rapeseed hinders our understanding of PCR. To explore rapeseed Cd-resistance mechanisms, we examined two genotypes, ‘ZS11’ (Cd-resistant) and ‘W10’ (Cd-sensitive), that exhibit contrasting PCR while having similar tissue Cd concentrations, and characterized their different fingerprints in terms of plant morphophysiology (electron microscopy), ion abundance (inductively coupled plasma mass spectrometry), DNA variation (whole-genome resequencing), transcriptomics (high-throughput mRNA sequencing), and metabolomics (ultra-high performance liquid chromatography-mass spectrometry). Fine isolation of cell components combined with ionomics revealed that more Cd accumulated in the shoot vacuoles and root pectins of the resistant genotype than in the sensitive one. Genome and transcriptome sequencing identified numerous DNA variants and differentially expressed genes involved in pectin modification, ion binding, and compartmentalization. Transcriptomics-assisted gene co-expression networks characterized BnaCn.ABCC3 and BnaA8.PME3 as the central members involved in the determination of rapeseed PCR. High-resolution metabolic profiles revealed greater accumulation of shoot Cd chelates, and stronger biosynthesis and higher demethylation of root pectins in the resistant genotype than in the sensitive one. Our comprehensive examination using a multiomics approach has greatly improved our understanding of the role of subcellular reallocation of Cd in the determination of PCR.

Keywords: Allotetraploid rapeseed, plant cadmium resistance, cadmium toxicity, multiomics, genotypic diversity, subcellular reallocation.
Cadmium (Cd) is a non-essential heavy metal that is highly bio-toxic and is easily diffused in the environment through industrial waste, sewage effluent, and agricultural run-off (Xue et al., 2014). Cd is absorbed mainly by plant roots and accumulates in edible tissues, thus posing a serious threat to human health via food chains (Touceda-González et al., 2015). To reduce the possible risk of excessive intake, various strategies have been developed to eliminate Cd from polluted soils, such as chemical sedimentation and chelation (Rizwan et al., 2018). However, these measures can potentially disrupt the soil structure and microbial activity (Dermont et al., 2008). In contrast, phytoremediation uses hyper-accumulator plants to remove pollutants from the environment and is considered to be a promising cost-effective and environmentally friendly remediation technology, although it does present a number of challenges (McGrath et al., 2006).

Previous studies have identified a number of model heavy metal-accumulating plants, including Sedum plumbizincicola, Arabidopsis helleri, and Noccaea caerulescens (Verbruggen et al., 2009; Peng et al., 2017). However, whilst these have strong metal accumulation they only produce relatively low biomass, which seriously restricts their practical use in the remediation of ecosystems. In contrast, oilseed rape (Brassica napus), a widespread oilseed crop (Blackshaw et al., 2011), shows great potential for phytoremediation by virtue of its large biomass production and strong metal accumulation (Grispen et al., 2006; Lacalle et al., 2018).

Enhanced plant Cd resistance (PCR), which is a complicated trait involving factors such as uptake, transport, compartmentation, and retention (Shahid et al., 2017), is a pivotal prerequisite for hyper-accumulation of excess Cd. Stabilization, chelation, and subsequent compartmentation of metal–ligand complexes to reduce cytosolic Cd represent several of the most important mechanisms underlying PCR. (Choppala et al., 2014). Previous studies have identified that the cell wall (CW) and vacuole play key roles in PCR (Sharma et al., 2016; Peng et al., 2017). The CW is the first barrier that prevents toxic heavy metals from entering root cells (Peng et al., 2017). Among the CW components, pectin, which is mainly regulated by pectin methyl esterase (PME) (Paynel et al., 2009), is a major contributor to CW-dependent retention of heavy metals (Peng et al., 2017). Phytochelatins (PCs) are a class of peptides comprising (Glu-Cys)n-Gly (n=2–11) and are synthesized by phytochelatin synthases (PCs) (Ogawa et al., 2011). PCs play pivotal roles in vacuolar Cd detoxification by chelating the metal with their thiol (-SH) groups (Ogawa et al., 2011). The transport of Cd–PC complexes into vacuoles is facilitated by ATP-binding cassette (ABC) transporters, such as ABCC1–3. (Song et al., 2010; Brunetti et al., 2015). In addition, Cd sequestration within vacuoles also occurs via PC-independent pathways: in these, direct Cd²⁺ transport is facilitated by Cd²⁺/proton (H⁺) anti-porters driven by trans-membrane H⁺ gradients (Khoudi et al., 2012). Heavy metal ATPase 3 (HMA3), cation/H⁺ exchangers (CAx) and metal transporter proteins (MTPs), which are tonoplast-localized, play key roles in the sequestration of Cd²⁺ into vacuoles (Zhao et al., 2009; Ueno et al., 2010; Sui et al., 2019).

Although PCR strategies have been elucidated in model heavy metal-accumulating plants, the genetic and molecular mechanisms underlying resistance in B. napus remain elusive due to the complexity of the allotetraploid rapeseed genome (AₐA₉Å₅C₉C₉, ~1345 Mb, 2n=4x=38) (Chalhoub et al., 2014; Bayer et al., 2017; Sun et al., 2017). We therefore carried out the present study with the aims of (i) screening and characterizing rapeseed genotypes with extremely contrasting PCR, (ii) determining the morphophysiological, genomic, transcriptomic, and metabolic bases that underlie such contrasting PCR, and (iii) identifying elite resources of genes/metabolites that can be used for PCR improvement. Using an integrated analysis of morphophysiological data combined with ionomics, genomics, transcriptomics, and metabolomics, we identified the pivotal role of subcellular reallocation of Cd in determining PCR, together with genes that have potential as targets for the improvement of PCR.

Materials and methods

Plant material and growth conditions

A panel consisting of 196 accessions of Brassica napus was collected in order to assess natural variations in PCR. Uniform 7–d-old seedlings were transplanted into black plastic containers holding 10 l Hoagland solution (Zhang et al., 2014). At 10-d-old, seedlings were transferred to solutions containing 10 μM Cd. The seedlings were cultivated in a growth chamber with the following conditions: light intensity, 300–320 μmol m⁻² s⁻¹; temperature, 25/22 °C day/night; photoperiod 16/8 h light/dark; relative humidity, 70%.

Microscopy analysis of leaf and root ultrastructure

Root hairs of seedlings were examined using an Olympus SZX16 stereo-scopic microscope. Pieces of young leaves (~1 mm²) from the seedlings were examined using TEM (H-7650, Hitachi) for characterization of differences in cell morphologies, plasma membranes, and CWs. Leaf pieces were also examined using SEM (JSM-6390/1V, JEOL, Tokyo, Japan) to characterize stomatal morphology and density. Samples were prepared for electron microscopy according to the method of Pan et al. (2012) and at least 10 independent biological replicates were examined.

Determination of chlorophyll pigments, malondialdehyde, and proline

After seedlings had been exposed to 10 μM Cd (CdCl₂) for 3 d as described above, the leaf chlorophyll contents were evaluated using a chlorophyll meter (SPAD-502, Konica Minolta). Determination of chlorophyll concentrations was performed using the method described by Hua et al. (2017). Lipid peroxidation was indicated by malondialdehyde (MDA) concentrations via thiobarbituric acid determination (Garg et al., 2006). Proline concentrations were determined spectrophotometrically using the ninhydrin assay (Bates et al., 1973).

Cadmium, low-temperature, metabolic inhibitor, and glutathione treatments

The initial screening of the accession panel identified a range of PCR across the genotypes (see Results). On this basis, we selected Cd-sensitive ‘Westar 10’ (‘W10’) and Cd-resistant ‘Zhonghuang11’ (‘ZH11’) for further detailed study.

For the low-temperature experiment, control seedlings were maintained at a constant temperature of 25 °C and for the low-temperature
treatment they were maintained at 4 °C. Other conditions within the growth chamber were as detailed above. For the metabolic inhibitor treatment, 10-d-old seedlings were transferred to solutions containing both 10 μM Cd and 50 μM 2,4-dinitrophenol (DNP) at a constant temperature of 25 °C. For the glutathione (GSH) treatment, 10-d-old seedlings were transferred to solutions containing both 10 μM Cd and 1 mM GSH at a constant temperature of 25 °C (Nakamura et al., 2013).

Collection of xylem and phloem sap, and quantification of mineral elements

Samples were divided into roots and shoots and were oven-dried at 65 °C until a constant weight was achieved. The dried tissues were subsequently transferred to a HNO₃/HClO₄ mixture (4:1, v/v) at 200 °C until they were completely digested (Luo et al., 2018). The samples were then diluted with deionized water, and the concentrations of mineral elements were quantified by inductively coupled plasma mass spectrometry (ICP-MS; NexION™ 350X, PerkinElmer).

Xylem sap was collected as described previously (Wu et al., 2015; Feng et al., 2018). ‘Z11’ and ‘W10’ seedlings that had been treated with 10 μM Cd for 3 d were cut at 2 cm above the shoot–root junction. The exudates were collected for 2 h as xylem sap, the samples were weighed, and then stored at 4 °C until further analysis. Phloem sap was collected in accordance with the method of Nakamura et al. (2009), as follows. To prevent air bubbles entering the vasculature, we removed the cotyledons using a razor blade immersed in deionized water before individual leaves were detached at the petioles. The leaves collected from one rapeseed plant were pooled together and flushed of xylem sap by placing the petioles in a tube filled with 300 ml of deionized water and incubated in an illuminated growth chamber for 15 min before further incubation in darkness for 1 h. The petioles were then re-cut under 5 mM Na₂-EDETA (pH 7.5) under low light before placing them in fresh 5 mM Na₂-EDETA. The leaves where then incubated in darkness for 1 h in a high-humidity chamber. Cd concentrations in the xylem and phloem sap were measured as described above.

Fractionation of CW components and quantification of Cd

Extraction of CWs and isolation of CW pectin was performed in accordance with the method described by Zhu et al. (2015). Briefly, the shoots and roots of the seedlings were collected and ground to fine powder in liquid nitrogen. Crude CWs were extracted using ice-cold 75% alcohol, followed by acetone, methanol:chloroform (1:1, v/v), and methanol, and were then lyophilized and stored at 4 °C until use. The supernatant, which was collected via hot-water extraction, was collected as the pectin fraction. The Cd concentrations in the CW components were determined as described above.

Fourier-transform infrared spectrometry

The relative abundances of functional groups in the lyophilized CWs, including carboxyl (COO⁻) and hydroxyl (OH⁻) groups, were analysed using Fourier-transform infrared spectrometry (FTIR, Vertex 70, Bruker Optics, Ettlingen, Germany) as described by Zhou et al. (2017). For each sample, five biological replicates were examined under the same conditions.

Determination of sulfide concentrations and activities of glutathione S-transferase and pectin methyl esterase

Total thiol (-SH groups) were determined according to the method of Tamás et al. (2016) with minor modifications (He et al., 2013). GSH levels were measured using the o-phthalaldehyde (OPA) fluorescence derivatization method (Guan et al., 2018). The activity of glutathione S-transferase (GST, E.C. 2.5.1.18) was assayed using a spectrophotometer in accordance with the method of Habig and Jakoby (1981), with minor modifications (Ghelfi et al., 2011). Uronic acid concentrations within the pectin were assayed according to the method of Blumenkrantz and Asboe-Hansen (1973) using galacturonic acid (Sigma-Aldrich) as a standard. As described by Brummell and Labavitch (1997), acetone-insoluble CWs were sequentially extracted with trans-1,2-diamino cyclohexane-N, N', N'', N'''-tetraacetic acid (CDTA) and Na₂CO₃ that each contained 0.1% NaBH₄ to isolate ionically bound and covalently bound pectin (Brummell et al., 2004). The activity of pectin methyl esterase (PME, E. C. 3.1.1.11) was measured at 25 °C (pH 7.5) using 0.2% pectin diluted in 0.1 M NaCl as previously described (Gaffe et al., 1992). The amount of methanol produced during the reaction was monitored using the alcohol oxidase method of Klovns and Bennet (1986).

Isolation of intact protoplasts and vacuoles, and measurement of Cd²⁺

Fresh leaves were used to isolate intact protoplasts and vacuoles as previously described by Robert et al. (2007). The purified protoplasts and vacuoles were used to determine Cd²⁺ concentrations and marker enzyme activity as described previously (Ma et al., 2005). The Cd concentrations were measured by ICP-MS as described above (Gong et al., 2003; Li et al., 2010).

Whole-genome resequencing

Fresh leaves of 10-d-old plants were sampled for isolation of genomic DNA (gDNA). An Illumina HiSeq 4000 system (read length 150 bp, paired end) belonging to the Novogene Biotechnology Company (Beijing, China) was used to perform whole-genome resequencing to distinguish variations in the gDNA. Genome-wide single-nucleotide polymorphisms (SNPs), insertions/deletions (Indels), copy number variations (CNVs), and structure variations (SVs) were identified and characterized between ‘Z11’ and ‘W10’ according to the method of Hua et al. (2018a).

Transcriptome sequencing

To identify the key genes regulating the differential responses to Cd toxicity between the genotypes, we performed high-throughput mRNA transcriptome sequencing on leaves and roots under both Cd-free and Cd conditions. Seedlings of ‘Z11’ and ‘W10’ were grown hydroponically in Cd-free solution for 10 d and then transferred to either fresh Cd-free (control) or 10 μM Cd (treatment) solutions for 6 h. The plants were then sampled (three biological replicates) and divided into root and shoot tissues. Total RNA of each sample was extracted using pre-chilled TRIzol reagent (Invitrogen) following the manufacturer’s instructions. A total of 48 RNA samples were processed using an Illumina Hiseq X Ten platform (Novogene, Beijing, China), which generated ~6.0 Gb of sequencing data with 150-bp paired-end reads for each sample.

Identification and characterization of differentially expressed genes

High-quality clean reads were mapped to the B. napus transcriptome reference of ‘Z11’ (Sun et al., 2017). The mRNA abundances of the unigenes were then identified using TopHat (http://ccb.jhu.edu/software/tophat/index.shtml) and Cufflinks (http://cole-trapnell-lab.github.io/cufflinks/) (Trapnell et al., 2012) and were normalized as fragments per kilobase of exon model per million mapped reads (FPKM) (Trapnell et al., 2010). The differentially expressed genes (DEGs) were defined as those with both a F-value and false-discovery rate less than 0.05 (Secco et al., 2013). Multiexperiment Viewer (MeV; http://www.tm4.org/#/welcome) (Eisen et al., 1998) was used to construct heatmaps based on the RNA-seq results. Gene ontology (GO) analyses of the DEGs were performed using the PANTHER classification system (http://www.pantherdb.org/data/) (Mi et al., 2005).
Identification and characterization of the metabolome

For the metabolomics analysis, the seedlings of ‘Z11’ and ‘W10’ were grown hydroponically under Cd-free condition for 10 d and were then transferred to solutions containing 10 μM Cd for 3 d before sampling (eight independent biological replicates). Leaf and root tissues were ground in liquid nitrogen and kept frozen until use. The frozen powder (~100 mg) was re-suspended in 1.0 ml of a mixture of pre-chilled CH₃OH:CH₃CN:H₂O (2:2:1, v:v:v) and then sonicated for 15 min (4 °C). The samples were vortexed for 30 s, stored on ice for 1 h, and then centrifuged (20 min at 14 000 g, 4 °C). The methanolic phases were recovered, diluted in 1:1 CH₃OH:CH₃CN, passed through 0.2-μm Minisart RC4 filters (Sartorius-Stedim Biotech, Gottingen, Germany), and then analysed using ultra-high performance liquid chromatography (UHPLC) with an Agilent 129 Infinity LC.

The UHPLC instrument was coupled to an ion-trap MS equipped with an electrospray ionization (ESI) source (Triple TOF 6600, Applied Biosystems Inc.), which was supplied with Shanghai Applied Protein Technology Co., Ltd. (Shanghai, China). The ESI sources were set according to the method described by Corso et al. (2018). Metabolites were characterized by comparing their retention times, m/z values, and fragmentation patterns with those of previous studies (Corso et al., 2018). The chromatograms were converted to net CDF files for peak alignment and peak area extraction using MZmine (http://mzmine.github.io/). Differential metabolites were defined as those with $P<0.05$.

cDNA synthesis and quantitative real-time PCR assays

Quantitative real-time PCR (qRT-PCR) assays of 10 randomly selected genes were used to verify the accuracy of the RNA-seq results according to a previously described protocol (Hua et al., 2018b). After treatment of RNA samples with RNase-free DNase I, the total RNA of fresh tissues was used as the templates for cDNA synthesis with a PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa). The qRT-PCR assays were performed using a SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (TaKaRa) kit under an Applied Biosystems StepOne™ Plus Real-time PCR system (ThermoFisher Scientific). The thermal cycle regimes were as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, and 60 °C for 30 s. A melt-curve analysis was also conducted as follows to ensure the primer-specificity of the target genes: 95 °C for 15 s, 60 °C for 1 min, and 60–95 °C for 15 s (+0.3 °C per cycle). The expression data were normalized using the public reference genes BnaEF1-α (Maillard et al., 2016) and BnaGD11 (Yang et al., 2014) with the $2^{-ΔΔC_T}$ method (Livak and Schmittgen, 2001). Each sample included three independent biological replicates. Gene-specific primers for qRT-PCR are listed in Supplementary Table S1 at JXB online.

Statistical analysis

Significant differences were determined using Student’s t-test or one-way ANOVA followed by Tukey’s honestly significant difference multiple comparison tests using the Statistical Productions and Service Solutions 17.0 toolkit (SPSS).

Data availability

The raw data for the whole-genome re-sequencing, mRNA transcriptome sequencing, and UHPLC-MS metabolome profiles have been deposited with the Bioproject ID 'PRJCA001323' in the BIG Data Center (http://bigd.big.ac.cn/) at the Beijing Institute of Genomics.

Results

Natural variations in Cd resistance among rapeseed genotypes

To identify genotypic differences in PCR among natural rapeseed genotypes we tested a panel of 196 accessions grown with an excess of Cd (10 μM Cd) in hydroponic culture, using the leaf chlorophyll concentration as a proxy for PCR. We observed that chlorophyll in young leaves (represented by SPAD values) ranged from 6.0 to 40.1 in the presence of Cd, and the values were normally distributed and had a coefficient of variation of 32.6% (Fig. 1A). This indicated that a wide natural variation in PCR occurred among the genotypes.

Fig. 1. Natural variations in plant cadmium (Cd) resistance and morphophysiological identification of the resistant and sensitive rapeseed genotypes. (A) Natural variations of Cd resistance in a panel comprising 196 accessions, as represented by chlorophyll SPAD values of young leaves. The SPAD values of the resistant genotype ‘Zhongshuang 11’ (‘Z11’) and the sensitive genotype ‘Westar 10’ (‘W10’) are indicated by arrows. (B) Growth performance of ‘Z11’ and ‘W10’ under Cd-free (control) and 10 μM Cd (CdCl₂) conditions. (C, D) Images of young leaves after exposure of plants Cd for 3 d (C) and 5 d (D). Leaf necrotic spots are indicated by arrows in (D), (E, F) Stem trichomes (E) and root hairs (F) of ‘Z11’ and ‘W10’ under control and 10 μM Cd conditions. All plants were grown hydroponically under Cd-free conditions for 10 d, and then transferred to 10 μM Cd. (This figure is available in colour at JXB online.)
Morphophysiological characterization of genotypes sensitive and resistant to Cd

In order to examine the biological basis of the natural variations observed in PCR, we focused on a Cd-resistant genotype, ‘Zhonghuang 11’ (‘Z11’), which is an elite cultivar with high seed yield and oil content (Sun et al., 2017), and a Cd-sensitive genotype, ‘Westar 10’ (‘W10’), which is highly susceptible to biotic and abiotic stresses (Kaur et al., 2009; Hua et al., 2016).

Under Cd-free (control) conditions, no marked differences in growth performance were observed between the two genotypes (Fig. 1B); however, when the plants were exposed to 10 μM Cd for 3 d, young leaves of ‘W10’ showed much more severe chlorosis than did those of ‘Z11’ (Fig. 1C). After a further 5 d of Cd treatment, we observed more necrotic spots on the young leaves of ‘W10’ than in those of ‘Z11’ (Fig. 1D). ‘Z11’ had more stem trichomes under Cd treatment (Fig. 1E, Supplementary Fig. S1A). We also noticed that the mean lengths of the root non-hair zones of both genotypes were clearly decreased under Cd toxicity (Fig. 1F); however, the root hairs of ‘Z11’ were much longer than did those of ‘W10’ (Fig. 1F; Supplementary Fig. S1B).

To examine the cellular effects underlying the morphological differences, we characterized the ultrastructure of the young leaves and root tips using electron microscopy. Compared with control conditions, the stomatal density of both genotypes significantly decreased under Cd toxicity (Fig. 2A–D). However, the stomatal density of the sensitive genotype ‘W10’ decreased to a greater degree than that of ‘Z11’ (Fig. 2E). Moreover, we also observed that Cd toxicity had a more pronounced effect on stomatal size in ‘W10’ than in ‘Z11’ (Fig. 2F–I, Supplementary Fig. S1C). The cell morphology of ‘Z11’ under Cd toxicity remained similar to that under Cd-free conditions (Fig. 2J, L, N); in contrast, the chloroplasts of ‘W10’ leaves were severely damaged by Cd, which also caused detached cells, swollen CWs, shrunk plasma membranes, and fewer starch grains (Fig. 2K, M, O). In the roots, we found that, compared with ‘W10’, the cells of ‘Z11’ had more pectosomes (Fig. 2P–S, Supplementary Fig. S1D), which may play key roles in scavenging of reactive oxygen species (ROS) induced by Cd toxicity.

Ionomnic characterization of genotypes sensitive and resistant to Cd

In addition to the disruption of leaf ultrastructure and root morphology, we also observed that Cd caused leaf chlorosis in both ‘Z11’ and ‘W10’. The chlorophyll a and b concentrations in the young leaves of ‘W10’ were significantly lower than in ‘Z11’ following exposure to Cd toxicity (Fig. 3A). Significantly greater MDA and proline concentrations were found in young leaves and in roots of ‘W10’ compared with ‘Z11’ as a result of Cd toxicity (Fig. 3B, C), indicating that ‘W10’ experienced more severe lipid peroxidation and proteolysis. Under short-term but high Cd exposure, the dry biomasses of the shoots and roots did not differ significantly between the two genotypes (Fig. 3D). We used ICP-MS to determine the tissue and subcellular concentrations of Cd$^{2+}$ and other cations, and found that the overall Cd concentration in the whole plants did not differ between ‘W10’ and ‘Z11’ (Fig. 3E).

Metal ions can be absorbed by plant roots both passively and actively (Zhao et al., 2002). To determine whether Cd accumulation differed between the symplastic and apoplastic pathways, we investigated the effects of low temperature (4 °C) (Feng et al., 2018) and a metabolic inhibitor DNP (Wolterbeek et al., 1988) on accumulation, since the symplastic pathway can be assumed to be minimal under these conditions (Zhao et al., 2002). Low temperature and addition of DNP both significantly inhibited Cd uptake in ‘Z11’ and ‘W10’, and the inhibitory effect was similar between the two genotypes (Fig. 3E). Given that net symplastic uptake can be estimated by subtracting the uptake at 4 °C or under DNP from that of the control (Cd-free, 25 °C), these results indicated that both the symplastic and apoplastic accumulation of Cd were similar between the Cd-resistant and Cd-sensitive genotypes. The Cd concentrations in both the xylem and phloem were also not significantly different between ‘Z11’ and ‘W10’ (Fig. 3F, G). We examined the Cd concentrations in young and mature leaves, and in the roots, but we did not still detect any significant differences between the genotypes (Fig. 3H). In addition, we also measured the tissue concentrations of Mg$^{2+}$, Mn$^{2+}$, Fe$^{2+}$, Cu$^{2+}$, Zn$^{2+}$, and Ca$^{2+}$. Although the concentrations of some ions, such as Mn$^{2+}$, Fe$^{2+}$, and Cu$^{2+}$, differed between the two genotypes under Cd-free conditions, most of the ion concentrations did not obviously differ under Cd toxicity (Supplementary Fig. S2).

The similar Cd concentrations between ‘Z11’ and ‘W10’ in both the whole plants and within individual tissues prompted us to investigate the subcellular reallocation of Cd within the CWs and vacuoles. Interestingly, we observed that the CW Cd concentration was significantly higher in the roots of ‘Z11’ than those of ‘W10’. No differences were detected in the concentrations within leaf CWs (Fig. 3I). We further divided the root CWs into pectin and other components, including cellulose and lignin among others, and found that pectin was mainly responsible for the differences in the Cd concentrations (Fig. 3J). By isolating leaf protoplasts and vacuoles, we found that the vacuole Cd concentration was significantly higher in ‘Z11’ than in ‘W10’ (Fig. 3K). A greater proportion (97.5%) of leaf protoplast Cd was reallocated to the vacuole in ‘Z11’ than that in ‘W10’ (91.8%) (Fig. 3L).

Taken together, these results suggested that shoot vacuolar sequestration and root CW retention of Cd might be the main causes of the differences in PCR between the resistant genotype ‘Z11’ and the sensitive genotype ‘W10’.

Genomic variations between the Cd-resistant and Cd-sensitive genotypes

To identify the genomic variations between the Cd-resistant and Cd-sensitive genotypes, we performed high-throughput whole-genome resequencing of ‘W10’, generating a total of ~40.0 Gb (>30×depth) data (Supplementary Table S2); previously the genome of ‘Z11’ has been de novo sequenced and released (Sun et al., 2017).

Based on the sequencing data, we characterized a total of 1 761 449 SNPs, 345 180 InDels, 71 641 SVs, and 40 415
CNVs in the rapeseed genome (A_n subgenome: A1–A9; C_n subgenome: C1–C9) (Fig. 4A, B). The genome-wide SNPs were unevenly distributed across the 19 chromosomes of B. napus, ranging from 37 368 (chr. A8) to 140 319 (chr. C3) with a mean of 92 708 SNPs on each chromosome (Supplementary Fig. S3A). The nucleotide diversity, π (mean SNP number per nucleotide), ranged from $1.45 \times 10^{-3}$ (chr. C5) to $3.97 \times 10^{-3}$ (chr. A2), with mean values of $\pi = 2.86 \times 10^{-3}$ (A_n subgenome) and $\pi = 1.90 \times 10^{-3}$ (C_n subgenome) (Fig. 4C, Supplementary Fig. S3B). The InDel number ranged from 8090 (chr. A8) to 27 387 (chr. A3) with a mean of 18 167 InDels on each chromosome (Supplementary Fig. S3C). We then used the reference of the ‘Zhonghuang 11’ (‘Z11’) genome annotation (Sun et al., 2017) to examine the genomic distribution of SNPs and InDels. Most of them were identified in the 2.0-kb upstream (promoter) and other intergenic regions (Fig. 4D), which indicated a crucial role of genomic variations in transcriptional regulation. The SVs affecting >50-bp genomic alterations were divided into five terms: genomic fragment insertion (109; 0.152%), deletion (18 573; 25.9%), inversion (1746; 2.44%), intra-chromosomal translocation (8943; 12.5%), and inter-chromosomal translocation (42 270; 59.0%) (Fig. 4E). CNVs belong to a class of SVs with variable copy numbers (Yu et al., 2011); whilst more genomic regions with
deleted copy numbers were found in ‘W10’, the total length of CNVs was similar between ‘Z11’ and ‘W10’ (Fig. 4F).

The genomic variants between ‘Z11’ and ‘W10’ were subjected to enrichment analysis of GO terms, which were grouped into the following three categories: ‘biological process’ (BP), ‘cellular component’ (CC), and ‘molecular function’ (MF) (Fig. 4G). In the MF annotations, the activities of metal-ion binding and transporters were most notable; in the CC annotations, the organelle and membrane parts were more prominent; and in the BP annotations, response to stimulus, signal transduction, and glutathione metabolism were highly enriched (Fig. 4G). The KEGG pathway analysis showed genetic variants involving metabolism of sulfide, including serine, cysteine, and methionine, and sugar were over-represented (Fig. 4H).

**Differential transcriptional responses to Cd toxicity between the resistant and sensitive genotypes**

After the removal of low-quality and adaptor sequences, a total of 423 million clean reads were obtained with a mean of ~53 million reads (~7.94 Gb of data) for each sample (Supplementary Table S3). To examine the accuracy of the RNA-seq data, we selected 10 genes to detect their expression levels via qRT-PCR assays. The results showed that most of the gene expression was highly consistent ($R^2$>0.95) between the transcriptome sequencing and the qRT-PCR validation (Fig. 5A), indicating high reliability of the sequencing results.

We found more genes responsive to Cd in the roots than in the shoots with a total of 315 and 3268 DEGs being identified.
Fig. 4. Genome-wide identification and molecular characterization of genomic DNA polymorphisms between the Cd-resistant rapeseed genotype ‘Z11’ and the Cd-sensitive genotype ‘W10’. (A) Overview of the genetic variants between the genotypes, as delineated by the Circos software (http://circos.ca/). In the figure, the genomic variants are as follows, outside-to-inside: (i) chromosomes, (ii) single nucleotide polymorphisms (SNPs), (iii) insertions/deletions (InDels), (iv) copy number variation (CNV) duplications, (v) CNV deletions, (vi) structure variation (SV) insertions, (vii) SV deletions, and (viii) intra-/inter-chromosomal translocation (SV inversion). (B) Number of the genome-wide genetic variants. (C) Nucleotide diversity ($\pi$) of the An and Cn sub-genomes. (D) Genomic annotation of SNPs and InDels. (E) Structural variation (SV). (F) Copy number variation (CNV). (G) Over-representation of GO terms. (H) Enrichment of KEGG pathways.
in the shoots and roots, respectively, between ‘Z11’ and ‘W10’ (Fig. 5B, C). A Pearson coefficient analysis showed low correlation between the shoot and root samples of both ‘Z11’ and ‘W10’ (Fig. 5D), indicating that they had distinct response patterns. The physiological data had indicated that shoot vacuolar sequestration and root CW retention of Cd might be the main causes of the differences in PCR between the two genotypes (Fig. 2). In terms of the KEGG pathways of the DEGs, we paid more attention to the genes involved in sulfur metabolism and ABC transporters that were enriched in the shoots (Fig. 5E); in the roots, we focused on the pathways implicated in oxidative phosphorylation and galactose metabolism that were over-represented (Fig. 5F).

The determination of PCR encompasses multiple processes involving uptake, translocation, and sequestration, among others (Corso et al., 2018; Fig. 6A). Our ionomics analysis revealed that Cd uptake did not significantly differ between the Cd-resistant and Cd-sensitive genotypes (Fig. 3); consistent with this finding, we also did not detect differential expression of the genes involved in Cd uptake, including Natural resistance-associated macrophage protein 5 (NRAMP5) (Fig. 6B), Zinc/Iron regulated proteins (ZIPs), and Yellow-stripe like proteins (YSLs) (data not shown). In the shoots, we focused on the DEGs involved in the vacuolar sequestration of dissociative Cd\(^{2+}\) and Cd chelates (Fig. 6C–M). Among the 16 DEGs for DEGs involved in the vacuolar sequestration of dissociative Cd\(^{2+}\) and Cd chelates (Fig. 6C–M), we characterized BnaA8.PME3 as the central gene involved in the regulation of pectin demethyl esterification (Fig. 7B). In turn, we found that the expression of BnaA8.PME3 in the roots of ‘Z11’ was ~26-fold higher than that of ‘W10’ under Cd toxicity (Fig. 7A), which was also confirmed by the qRT-PCR assay (Fig. 7C).

### Differential metabolic fingerprints in responses to Cd toxicity between the resistant and sensitive genotypes

Our physiological, genomic, and transcriptomic analyses had indicated that different Cd chelation and vacuolar sequestration, and CW retention were the main causes of the differences in PCR in the shoots and roots, respectively. To further confirm this, we employed UHPLC-MS to identify the different metabolites involved in Cd chelation and retention. Under Cd toxicity, the different genotypes and tissues exhibited significantly different metabolic features compared with the control (Fig. 8A), indicating genotype- and tissue-specific metabolic responses to Cd. Principal component analysis (PCA) showed that the sample distributions on PC1 (51.9% of the variance of the shoots and 42.1% of the variance of the roots) were determined mainly by the genotype (Fig. 8B, C).

Regardless of shoots or roots, more metabolites with higher abundance were detected in ‘Z11’ than in ‘W10’ (Fig. 8D, E). Metabolite enrichment analyses revealed that in the shoots, the metabolism of sulfur-containing compounds, such as serine, cysteine, methionine, and GSH, were highly enriched (Fig. 8F). In the roots, most of the differences in metabolites were related to phenylalanine/phenypropanoids as well as some saccharides, including galactose and fructose (Fig. 8G), which play crucial roles in CW biosynthesis. Detailed metabolite fingerprints were confirmed by heatmaps showing the signals of all the isotopes for each metabolite (Fig. 9). In addition to higher levels of numerous amino acids and organic acids that act as key Cd\(^{2+}\) ligands, higher levels of sulfide (including serine, cysteine, GSH, and methionine) and nicotinamide compounds (Fig. 9A) indicated stronger Cd chelation, subsequently followed by vacuolar sequestration, which occurred more in the shoots of resistant ‘Z11’ than in those of sensitive ‘W10’. By summarizing of the pectin biosynthesis pathway (Fig. 9B), we found that some pectin synthesis substrates accumulated more highly in the roots of ‘Z11’ than in those of ‘W10’ (Fig. 9C).

Using a chemical colorimetry method, we confirmed that the concentrations of total sulfhydryl (-SH), cysteine, and GSH in the shoots of ‘Z11’ were indeed significantly higher than those of ‘W10’ (Fig. 10A–C), indicating high accuracy of our metabolomics data. The activity of GST, which mediates the binding of GSH to cytotoxic compounds (Ghelfi et al., 2011), was stronger in the shoots of ‘Z11’ than in those of ‘W10’ (Fig. 10D). Moreover, exogenous addition of GSH clearly alleviated...
the leaf chlorosis in ‘W10’ caused by Cd toxicity (Fig. 10E), which might be attributed to a GSH-induced reduction of Cd absorption *in vitro* and efficient chelation with Cd *in vivo* (Nakamura et al., 2013). In the roots, although the concentrations of covalently bound pectin were similar between the two genotypes (Fig. 10F), we observed that ‘Z11’ had more ionically bound pectin (Fig. 10G) and uronic acid (Fig. 10H) than ‘W10’. In addition, ‘Z11’ also showed higher PME activity (Fig. 10I). These results were further validated by the higher degree of pectin demethylation in ‘Z11’ than in ‘W10’, which
was reflected by the higher abundance of carboxyl (COO\(^-\)) and hydroxyl (OH\(^-\)) groups (Fig. 10J).

**Discussion**

As a staple oilseed crop species that produces large amounts of biomass, *B. napus* has a great potential for the phytoremediation of soils polluted with Cd (Grispen et al., 2006). Improving plant Cd resistance (PCR), which contributes to its hyper-accumulation, is a key prerequisite for plant-assisted phytoremediation. However, the regulatory mechanisms that underlie PCR in rapeseed remain elusive due to the complexity of its allotetraploid genome. In this study, by assessing PCR, we identified a Cd-resistant genotype ‘Z11’ and a Cd-sensitive genotype ‘W10’ from a rapeseed panel comprising 196 accessions (Fig. 1). By further performing an integrated analysis of...
morphophysiological, ionicomic, genomic, transcriptomic, and metabolomics data, we gained a comprehensive understanding of the different responses to Cd toxicity and the PCR strategies.

Pivotal roles of subcellular reallocation of Cd in the determination of PCR

We did not observe significant differences in either shoot or root Cd concentrations between the resistant and sensitive genotypes (Fig. 3E). By isolating individual cell components, we found that vacuolar compartmentalization of Cd in the shoots and retention of Cd in cell wall (CW) pectin in the roots might be the main causes of the differences in PCR (Figs 3I–L, 11), which suggested that different strategies for PCR exist between different plant tissues.

For most plants, Cd tends to accumulate more in the roots than in the shoots (Song et al., 2017), which was also observed in the present study (Fig. 3H). The concentrations in the roots were 4-fold higher than those in the shoots, which suggested that the root endodermis and xylem might serve as an effective barrier for root-to-shoot translocation. The plant root CWs, which are composed of polysaccharides and proteins, function as the first protective barrier in defense against biotic and abiotic threats (Gutsch et al., 2018). The efficacy of roots in terms of Cd retention is mainly attributable to the number of available functional groups (e.g. carboxyl and hydroxyl) in the CWs that can bind with heavy metal cations (Nishizono et al., 1987). Among the CW components, pectin, which is regulated mainly by PME (Paynel et al., 2009), is a major contributor to Cd retention (Peng et al., 2017). We found that differential reallocation within pectin was mainly responsible for the distinct Cd concentrations in the root CWs (Fig. 3J), which highlighted the crucial role of pectin in the determination of PCR.

In addition to the CW retention of Cd, vacuolar compartmentalization is also pivotal for enhanced PCR because it restricts the mobility of free Cd in the cytosol (Shahid et al., 2017). In the present study, we found that a larger proportion of protoplast Cd was reallocated into the shoot vacuoles in resistant ‘Z11’ compared with sensitive ‘W10’ (Fig. 3K, L). The chelation of Cd\textsuperscript{2+} by sulfur-containing compounds, particularly GSH and its-derived PCs, plays a key role in its subsequent
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vacuolar compartmentation (Han et al., 2019). Nakamura et al. (2013) reported that GSH applied to roots reduces Cd concentrations in the symplastic sap of root cells and significantly inhibits its root-to-shoot translocation via xylem vessels. GSH applied to roots also activates Cd efflux from root cells to the hydroponic solution. In addition, GSH application activates the antioxidant system to reduce Cd-induced accumulation of ROS and reduces damage to the ultrastructure of the cells (Seth et al., 2012). By integrating ultra-high performance liquid chromatography and a chemical colorimetry method, we confirmed that the concentrations of total sulydryl (-SH), cysteine, and GSH in the shoots of ‘Z11’ were indeed significantly higher than those of ‘W10’ (Figs 9, 10A–C), and exogenous addition of GSH to the roots clearly alleviated the leaf chlorosis in ‘W10’ caused by Cd toxicity (Fig. 10E). The GSH-mediated enhancement of PCR may be attributed to reduced Cd absorption in vitro, efficient chelation with Cd in vivo, and GSH activation of ROS scavenging.

Overall, efficient retention of Cd by root CWs combined with shoot vacuolar sequestration contributed synergistically to strengthening PCR (Fig. 11), which highlights the pivotal role of subcellular reallocation of Cd in determining PCR.

Fig. 8. Metabolic characterization of differential metabolites between the Cd-resistant rapeseed genotype ‘Z11’ and the Cd-sensitive genotype ‘W10’.

(A) Quality control of the differential metabolite charges in the shoots and roots between the two genotypes under Cd toxicity. The values of metabolic profiles under Cd toxicity were calculated relative to the control. (B, C) Principal component analysis of differential metabolites in the shoots (B) and roots (C). (D, E) Volcano diagrams showing differential metabolites in the shoots (D) and roots (E). ‘Z11 > W10’ indicates the metabolites had higher accumulation in ‘Z11’ than in ‘W10’, and vice versa. (F, G) Metabolic pathway enrichment analysis of the differential metabolites in the shoots (F) and roots (G) between ‘Z11’ and ‘W10’. The circle size indicates the degree of enrichment. Metabolites were determined by high-throughput HPLC-MS. The plants were grown hydroponically under Cd-free conditions for 10 d, and were then transferred to 10 μM Cd for 3 d before being sampled. (This figure is available in colour at JXB online.)
Using a multiomics approach to elucidate the mechanisms underlying PCR

Many previous studies of PCR have focused on changes in plant metabolism (Wu et al., 2015; Yan et al., 2016), the transcriptome (Zhou et al., 2012; Jian et al., 2018), or the proteome (Marmiroli et al., 2015), and have failed to provide a systematic understanding of plant responses to Cd toxicity. Currently, an increasing number of omics resources are being broadly applied across the life sciences as a result of the comprehensive insights that they are capable of providing. For example, comparative transcriptome sequencing combined with morphophysiological analyses in sweet sorghum have revealed key factors that affect differential Cd accumulation in two contrasting genotypes (Feng et al., 2018). In Arabidopsis halleri, integrated RNA-seq and metabolomics profiles have been analysed to reveal contrasting PCR strategies in two metalloclous populations (Corso et al., 2018). However, few multiomics studies, particularly those involving PCR, have been conducted so far on allotetraploid rapeseed.

In the present study, we first identified many differences in plant ultrastructural morphology, including in root hairs, stomatal size and number, chloroplasts, trichomes, and peroxisomes (Figs 1, 2, Supplementary Fig. S1). Excess Cd significantly inhibits root growth (Han et al., 2019) and results in swelling in leaves and disorganization of the chloroplast stroma/granum lamellae, smaller stomatal perimeters, and an increased number of closed stomata (Li et al., 2017). Thus, root performance and the ultrastructure of chloroplasts and stomata may be used to indicate the resistance or sensitivity of plants to Cd toxicity, and hence the longer root hairs, well-organized chloroplasts, and larger stomata of ‘Z11’ might be due to its more enhanced PCR compared with ‘W10’. These differences in growth performance may well affect essential nutrient absorption and photosynthesis. One of the most important functions of trichomes in plants is the sequestration and compartmentalization of heavy metals through active secretion of metal crystals (Choi et al., 2001; Sarret et al., 2002; Broadhurst et al., 2004; Harada et al., 2010). The structure of chloroplasts and stomata are also used as an indicator of PCR. Excessive Cd concentrations usually cause over-production of ROS, which may result in cell death due to oxidative processes, such as damage to nucleic acid and peroxidation of membrane lipids (Shahid et al., 2017).

To minimize Cd-induced ROS formation, plants usually activate antioxidants produced by peroxisomes to act as scavengers (Charton et al., 2019). We observed more peroxisomes in ‘Z11’ than in ‘W10’, which might contribute to enhanced PCR of the former through efficiently reducing Cd-induced ROS accumulation. Taken together, the differences in leaf trichome numbers, chloroplast organization, and stomatal apertures lead us to propose that they are involved in the differences in PCR and Cd-induced growth effects between the two genotypes.

Fig. 9. Differential metabolite fingerprints in the shoots and roots between the Cd-resistant rapeseed genotype ‘Z11’ and the Cd-sensitive genotype ‘W10’. (A) Differential metabolite profiling in the shoots of the two genotypes under Cd toxicity. (B) Schematic diagram showing the pectin biosynthesis pathway. (C) Differential metabolite profiling in the roots. Metabolites were determined by high-throughput HPLC-MS. The plants were grown hydroponically under Cd-free conditions for 10 d, and were then transferred to 10 μM Cd for 3 d before being sampled. The concentration abundance was calculated as a percentage relative to the sample with the highest value for each metabolite. (This figure is available in colour at JXB online.)
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Our ionomics data suggested that shoot vacuolar sequestration and retention of Cd in root CW pectin were mainly responsible for the contrasting PCR between the resistant and sensitive genotypes (Fig. 3), indicating that there were different ion reallocation strategies within them. To uncover the genomic basis of the differential PCR, we performed whole-genome resequencing on the resistant and sensitive genotypes. Large numbers of the DNA variants between these rapeseed genotypes were mapped onto genes involved in metal binding and cell macromolecule biosynthesis, such as sulfur-containing compounds (serine and glutathione) and CW components (particularly sugar) (Fig. 4G, H), and hence these genes might be responsible for the stimulus response to Cd toxicity, vacuolar Cd sequestration, and pectin-mediated Cd retention. Further, our transcriptomics-assisted analysis revealed that in the shoots, the expression of genes involved in Cd chelation and vacuolar Cd compartmentalization (sulfur metabolism and ABC transporters) was significantly higher in ‘Z11’ than in ‘W10’ (Fig. 5).

Fig. 10. Validation of some key metabolites and enzymes in shoots and roots between the Cd-resistant rapeseed genotype ‘Z11’ and the Cd-sensitive genotype ‘W10’. (A–C) Concentrations of (A) total sulfydryl (-SH), (B) cysteine, and (C) glutathione (GSH) in the shoots between ‘Z11’ and ‘W10’ under Cd-free (control) and Cd conditions. (D) Activity of glutathione S-transferase (GST) in the shoots. (E) Images of leaves of plants with Cd toxicity with or without addition of GSH. The plants were grown hydroponically under Cd-free conditions for 10 d, and were then transferred to either 10 μM Cd (Cd-GSH) or 10 μM Cd supplemented with 1 mM GSH (Cd+GSH) for 3 d. (F–H) Concentrations of covalently bound pectin (F), ionically bound pectin (G), and uronic acid (H) in the root cell walls. (I) Pectin methyl esterase (PME) activity in the roots. (J) Relative abundance of carboxyl (COO⁻) and hydroxyl (OH⁻) in root pectins’. Apart from (E), the plants were grown hydroponically under Cd-free conditions for 10 d, and were then transferred to 10 μM Cd for 3 d before being sampled. Data are means (±SE), n=3. Different letters indicate significant differences as determined using ANOVA and Tukey’s HSD (P<0.05). (This figure is available in colour at JXB online.)
mRNA levels of PMEs in ‘Z11’ compared to ‘W10’ in the roots (Fig. 6) indicated that the CW pectin derived from carbohydrates might have a higher degree of demethylation. This potentially produces greater numbers of free functional groups with negative charges, which are essential for binding of cation ions and hence for binding and detoxification of Cd (Zhu et al., 2018). To further confirm our hypothesis, we used a UHPLC-MS platform to characterize the differential metabolites between the two genotypes. Detailed metabolic fingerprints showed that ‘Z11’ had more sulfide, organic acids, and amino acids in the shoots than ‘W10’ (Figs 8F, 9A, 10A–C), which showed weaker Cd chelation. The root metabolic profiling showed that ionically bound pectin and uronic acid accumulated less in ‘W10’ than in ‘Z11’ (Figs 8G, 9C, 10F–J), which had stronger pectin biosynthesis and higher degrees of demethylation.

**Potential utilization of ABC and PME genes in the genetic improvement of PCR**

In this study, we focused on the ABC transporter genes involved in the vacuolar sequestration of Cd and the PME genes involved in regulating CW pectin-mediated retention of Cd, both of which we found to play crucial roles in determining PCR.

The CAXs/HMA3 and ABC transporters mediate the transport of free Cd$^{2+}$ and Cd chelates, respectively, into vacuoles (Zhao et al., 2009; Song et al., 2010; Ueno et al., 2010; Brunetti et al., 2015). Only a small fraction of the Cd in the plant cytosol is present in the form of free Cd$^{2+}$, and most is bound to ligands (Callahan et al., 2006). Therefore, we paid more attention to the ABC transporters involved in Cd sequestration. In the model plants Arabidopsis and rice, more than 120 ABC family members have been identified, although only a few of them have been functionally characterized (Lefèvre et al., 2015). Thus far, eight ABC subfamilies, namely ABCA–G and ABCI, have been identified in plants (Zhang et al., 2018) but only three ABC (ABCC1/2/3) subgroup members have been demonstrated to mediate the sequestration of PC–Cd or GSH–Cd complexes into vacuoles (Salt and Rauser, 1995; Li et al., 1997; Song et al., 2010; Brunetti et al., 2015). However, redundancy in vacuolar sequestration of Cd occurs among the three ABC transporters (Brunetti et al., 2015), and the reason why the HMA3 gene,
but not the ABCC transporter genes, is always identified as the major determinant for Cd accumulation or PCR in genome-wide association studies is probably due to the large functional redundancy of ABCC1, ABCC2, and ABCC3, and to the low probability that all of these genes are mutated in the same plant (Zhang et al., 2018). In our present study, we did not detect any expression of either ABCC1 or ABCC2. Among the genome-wide ABCC3 family genes of rapeseed, six Cd-responsive members were differentially expressed between ‘Z11’ and ‘W10’, and BnaCn.ABCC3 was characterized as the central member that is potentially involved in Cd chelate sequestration into the vacuoles (Fig. 6). Although overexpression of the ABCC genes enhances PCR, their function is largely dependent on PC biosynthesis (Song et al., 2010; Brunetti et al., 2015). Thus, compared with separate overexpression of ABCCs and PCs, concurrent modulation of them may have a more pronounced effect on improvement of PCR.

PMEs determine pectin methyl esterification by regulating the number of free carboxyl and hydroxyl groups that have negative charges, which greatly affects trapping of Cd by CWs (Song et al., 2017). Suitably enhanced expression of PMEs and stronger PME activity contribute to a higher degree of pectin demethylation, which creates more binding sites for Cd within the CW (Gutsch et al., 2018). Overexpression of PME14 in rice significantly increases aluminum contents in root-tip CWs (Yang et al., 2013). A mutation in the Arabidopsis PME3 as well as its aberrant expression cause hypersensitivity specifically to excess zinc (Weber et al., 2013). Therefore, the potential exists to engineer the expression of PMEs in order to enhance deposition of Cd within CWs, thus further protecting the cytosol from toxicity, and ultimately increasing the PCR.

In conclusion, in this study we have integrated morphophysiological, ionomic, genomic, transcriptomic, and metabolomics data to systematically elucidate the key roles of vacuolar sequestration and cell wall pectin retention in rapeseed resistance to Cd toxicity (Fig. 11). We can also conclude that such an multiomics-assisted examination of crop traits provides a powerful tool for improving our understanding of regulatory mechanisms.

**Supplementary data**

Supplementary data are available at JXB online.

Table S1. Gene-specific primers used for qRT-PCR assays in this study.

Table S2. Overview of the whole-genome resequencing data of sensitive genotype ‘W10’.

Table S3. Overview of the transcriptome sequencing data of the Cd-resistant and Cd-sensitive genotypes.

Fig. S1. Trichome density, mean length of root hairs, stomatal size, and peroxisome numbers per cell under Cd toxicity in the Cd-resistant and Cd-sensitive genotypes.

Fig. S2. Tissue concentrations of several cations in the Cd-resistant and the Cd-sensitive genotypes.

Fig. S3. Characterization of single-nucleotide polymorphisms and insertions/deletions between the Cd-resistant and the Cd-sensitive genotypes.

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**References**

Bates LS, Waldren RP, Teare ID. 1973. Rapid determination of free proline for water-stress studies. Plant and Soil 39, 205–207.

Bayer PE, Hurgobin B, Golicz AA, et al. 2017. Assembly and comparison of two closely related Brassica napus genomes. Plant Biotechnology Journal 15, 1602–1610.

Blackshaw R, Johnson E, Gan YT, May W, McAndrew D, Barthet V, McDonald T, Wispinski D. 2011. Alternative oilseed crops for biodiesel feedstock on the Canadian prairies. Canadian Journal of Plant Science 91, 889–896.

Blumenkrantz N, Asboe-Hansen G. 1973. An improved method for the assay of hydroxylsine. Analytical Biochemistry 56, 10–15.

Broadhurst CL, Chanley RL, Angle JS, Maugel TK, Erbe EF, Murphy CA. 2004. Simultaneous hyperaccumulation of nickel, manganese, and calcium in Alysum leaf trichomes. Environmental Science & Technology 38, 5797–5802.

Brummell DA, Dal Cin V, Crisosto CH, Labavitch JM. 2004. Cell wall metabolism during maturation, ripening and senescence of peach fruit. Journal of Experimental Botany 55, 2029–2039.

Brummell DA, Labavitch JM. 1997. Effect of antisense suppression of endopolygalacturonase activity on polyuronide molecular weight in ripening tomato fruit and in fruit homogenates. Plant Physiology 115, 717–725.

Brunetti P, Zanella L, De Paolis A, Di Litta D, Cecchetti V, Falasca G, Barbieri M, Altamura MM, Costantino P, Cardarelli M. 2015. Cadmium-inducible expression of the ABC-type transporter AtABCC3 increases phytochelatin-mediated cadmium tolerance in Arabidopsis. Journal of Experimental Botany 66, 3815–3829.

Cai MZ, Wang FM, Li RF, Zhang SN, Wang N, Xu GD. 2011. Response and tolerance of root border cells to aluminum toxicity in soybean seedlings. Journal of Inorganic Biochemistry 105, 966–971.

Callahan DL, Baker AJ, Kolev SD, Wedd AG. 2006. Metal ion ligands in hyperaccumulating plants. Journal of Biological Inorganic Chemistry 11, 2–12.

Chalhoub B, Denoefu D, Liu S, et al. 2014. Early allopolyploid evolution in the post-Neolithic Brassica napus oilseed genome. Science 345, 950–953.

Charton L, Plett A, Linka N. 2019. Plant peroxisomal solute porter proteins. Journal of Integrative Plant Biology. In press. doi:10.1111/jipb.12790.

Choi YE, Harada E, Wada M, Tsuoboi H, Morita Y, Kusano T, Sano H. 2001. Detoxification of cadmium in tobacco plants: formation and active excretion of crystals containing cadmium and calcium through trichomes. Planta 213, 45–50.

Choppala C, Bolen N, Bibi S, Iqbal M, Rengel Z, Kuhnhrishnan A, Ashwath N, Ok YS. 2014. Cellular mechanisms in higher plants governing resistance to cadmium toxicity. Critical Reviews in Plant Sciences 33, 374–391.

Corso M, Schwartzman MS, Guzzo F, Souard F, Malkowski E, Hanikenne M, Verbruggen N. 2018. Contrasting cadmium resistance strategies in two metallocculuss populations of Arabidopsis halleri. New Phytologist 218, 283–297.

Dermont G, Bergeron M, Mercier G, Richer-Lafleche M. 2008. Soil washing for metal removal: a review of physical/chemical technologies and field applications. Journal of Hazardous Materials 152, 1–31.

Eisen MB, Spellman PT, Brown PO, Botstein D. 1998. Cluster analysis and display of genome-wide expression patterns. Proceedings of the National Academy of Sciences, USA 95, 14863–14868.
Lefèvre F, Baijot A, Boutry M. 2015. Plant ABC transporters: time for biochemistry? Biochemical Society Transactions 43, 931–936.

Li JY, Fu YL, Pike SM, et al. 2010. The Arabidopsis nitrate transporter NRT1.5 functions in nitrate removal from the xylem sap and mediates cadmium tolerance. The Plant Cell 22, 1633–1646.

Li M, Hao P, Cao F. 2017. Glutathione-induced alleviation of cadmium toxicity in Zea mays. Plant Physiology and Biochemistry 119, 240–249.

Li ZS, Lu YP, Zhen RG, Szczypka M, Thiele DJ, Rea PA. 1997. A new pathway for vacuolar cadmium sequestration in Saccharomyces cerevisiae: YCF1-catalyzed transport of bis(glutathionato) cadmium. Proceedings of the National Academy of Sciences, USA 94, 42–47.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression using real-time quantitative PCR and the 2−ΔΔCt method. Methods 25, 402–408.

Lozano-Rodriguez E, Hernandez LE, Bonay P, Carpena-Ruiz RO. 1997. Distribution of cadmium in shoot and root tissues. Journal of Experimental Botany 48, 123–128.

Luo JS, Huang J, Zeng DL, et al. 2018. A defensin-like protein drives cadmium efflux and allocation in rice. Nature Communications 9, 645.

Ma JF, Ueno D, Zhao FJ, McGrath SP. 2005. Subcellular localisation of Cd and Zn in the leaves of a Cd-hyperaccumulating ectotype of Thlaspi caerulescens. Planta 220, 731–736.

Maillard A, Etienne P, Diquétou S, Trouverie J, Billard V, Yvin JC, Querry A. 2016. Nutrient deficiencies modify the ionic composition of plant tissues: a focus on cross-talk between myo-inositol and other nutrients in Brassica napus. Journal of Experimental Botany 67, 5631–5641.

Marmiroli M, Imperiale D, Pagano L, Villani M, Zappettini A, Marmiroli N. 2015. The proteomic response of Arabidopsis thaliana to cadmium sulphide quantum dots, and its correlation with the transcriptomic response. Frontiers in Plant Science 6, 1104.

McGrath SP, Lombi E, Gray CW, Caille N, Dunham SJ, Zhao FJ. 2006. Field evaluation of Cd and Zn phytoextraction potential by the hyperaccumulators Thlaspi caerulescens and Arabidopsis halleri. Environmental Pollution 141, 115–125.

Mi HY, Lazareva-Ulitsky B, Luo R, et al. 2005. The PANTHER database of protein families, subfamilies, functions and pathways. Nucleic Acids Research 33, D284–D288.

Nakamura S, Maruyama K, Watanabe A, Hattori H, Chino M. 2005. Response of glutathione in the sieve tube of Brassica napus L. to cadmium treatment. In: K Salto, Lu De K, Stulen, MJ Hawkesford, E Schnug, eds. Sulfur transport and assimilation in plants in the post genomic era. Leiden, Netherlands: Backhuys Publishers, 229–232.

Nakamura S, Suzui N, Nagasaki T, et al. 2013. Application of glutathione to roots selectively inhibits cadmium transport from roots to shoots in oiled rape. Journal of Experimental Botany 64, 1073–1081.

Nishizono H, Ichikawa H, Suzuki S, Ishii F. 1987. The role of the root cell wall in the heavy metal resistance of Athyrium yokoscense. Plant and Soil 101, 15–20.

Ogawa S, Yoshidomi T, Yoshimura E. 2011. Cadmium(II)-stimulated enzyme activation of Arabidopsis thaliana phytochelatin synthase 1. Journal of Inorganic Biochemistry 105, 111–117.

Pan Y, Wang ZH, Yang L, Wang ZF, Shi L, Naran R, Azadi P, Xu FS. 2012. Differences in cell wall components and allocation of boron to cell walls confer variations in sensitivities of Brassica napus cultivars to boron deficiency. Plant and Soil 347, 438–394.

Park J, Song WY, Ko D, Eom Y, Hansen TH, Schiller M, Lee TG, Martinoa E, Lee Y. 2012. The phytochelatin transporters ABCC1 and ABCC2 mediate tolerance to cadmium and mercury. The Plant Journal 69, 278–288.

Paynel F, Schaumann A, Arkoun M, Douchiche O, Morvan C. 2009. Temporal regulation of cell-wall pectin methylsterase and peroxidase isoforms in cadmium-treated flax hypocotyl. Annals of Botany 104, 1363–1372.

Peng JS, Wang YJ, Ding G, Ma HL, Zhang YJ, Gong JM. 2017. A pivotal role of cell wall in cadmium accumulation in the grasses genotypes Sedum plumbizincicum. Molecular Plant 10, 771–774.

Rizwan M, Ali S, Zia ur Rehman M, Rinklebe J, Tsang DCW, Bashir A, Maqbool A, Tack FMG, Ok YS. 2018. Cadmium phytoextraction potential of B. napus genotypes: a review. The Science of the Total Environment 631–632, 1175–1191.
Robert S, Zouhar J, Carter C, Raikhel N. 2007. Isolation of intact vacuoles from Arabidopsis rosette leaf-derived protoplasts. Nature Protocols 2, 259–262.

Salt DE, Rauser WE. 1995. MgATP-dependent transport of phytochelatins across the tonoplast of oat roots. Plant Physiology 107, 1293–1301.

Sarret G, Saumitou-Laprade P, Bert V, Proux O, Hazemann JL, Traverse A, Marcus MA, Manceau A. 2002. Forms of zinc accumulated in the hyperaccumulator Arabidopsis halleri. Plant Physiology 130, 1815–1826.

Secco D, Jabnoune M, Walker H, Shou H, Wu P, Poirer Y, Whelan J. 2013. Spatio-temporal transcript profiling of rice roots and shoots in response to phosphate starvation and recovery. The Plant Cell 25, 4285–4304.

Seth CS, Remans T, Keenen E, Jozefczak M, Gielien H, Opdenakker K, Weyens N, Vangronsveld J, Cuypers A. 2012. Phytoextraction of toxic metals: a central role for glutathione. Plant, Cell & Environment 35, 334–346.

Shahid M, Dumat C, Khalid S, Niazi NK, Antunes PMC. 2015. Cadmium bioavailability, uptake, toxicity and detoxification in soil-plant system. Reviews of Environmental Contamination and Toxicology 241, 73–137.

Sharma SS, Dietz KJ, Mimura T. 2016. Vacuolar compartmentalization as indispensable component of heavy metal detoxification in plants. Plant, Cell & Environment 39, 1112–1126.

Song WY, Park J, Mendoza-Cózatl DG, et al. 2010. Arsenic resistance in Arabidopsis is mediated by two ABCC-type phytochelatin transporters. Proceedings of the National Academy of Sciences, USA 107, 21187–21192.

Song Y, Jin L, Wang X. 2017. Cadmium absorption and transportation pathways in plants. International Journal of Phytoremediation 19, 133–141.

Sui F, Zhao D, Zhu H, Gong Y, Tang Z, Huang XY, Zhang G, Zhao FJ. 2019. Map-based cloning of a new total loss-of-function allele of OsHMA3 causes high cadmium accumulation in rice grain. Journal of Experimental Botany 70, 2857–2871.

Sun F, Fan G, Hu Q, et al. 2017. The high-quality genome of Brassica napus cultivar Z511 retrospect introgression history in semi-winter morphotype. The Plant Journal 92, 452–468.

Tamás L, Mistrik I, Zelínová V. 2016. Cadmium activates both diphenylethenolidion- and rotenone-sensitive superoxide production in barley root tips. Planta 214, 1277–1287.

Trapnell C, Roberts A, Goff L, Pertea G, Kim D, Kelley DR, Pimentel H, Salzberg SL, Wold BJ, Pachter L. 2012. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nature Protocols 7, 562–578.

Trapnell C, Williams JA, Pertea G, Mortazavi A, Watanabe K, Gentzis K, Neph S,afneus LM, Afshar RM, Lee W, et al. 2010. Transcript assembly and quantification of RNA-seq experiments with TopHat. Genome Biology 11, R20.

Trappe H, Dorn Y, Wu X, Zhao J, Li T, Zhu Y, Zheng X, et al. 2015. Cadmium absorption and transportation pathways in plants. International Journal of Phytoremediation 19, 133–141.

Ueno D, Yamaji N, Kono I, Huang CF, Ando T, Yano M, Ma JF. 2015. Xylem transport and gene expression play decisive roles in cadmium accumulation in shoots of two oilseed rape cultivars (Brassica napus). Chemosphere 119, 1217–1223.

Xue D, Jiang H, Deng X, Zhang X, Wang H, Xu X, Hu J, Zeng D, Guo L, Qian Q. 2014. Comparative proteomic analysis provides new insights into cadmium accumulation in rice grain under cadmium stress. Journal of Hazardous Materials 280, 269–278.

Yan H, Filardo F, Xu H, Zhao X, Fu D. 2016. Cadmium stress alters the redox reaction and hormone balance in oilseed rape (Brassica napus L.) leaves. Environmental Science and Pollution Research International 23, 3758–3769.

Yang H, Liu J, Huang S, Guo T, Deng L, Hua W. 2014. Selection and evaluation of novel reference genes for quantitative reverse transcription PCR (qRT-PCR) based on genome and transcriptome data in Brassica napus L. Gene 538, 113–122.

Yang XY, Zeng ZH, Yan JY, Fan W, Bian HW, Zhu MY, Yang JL, Zheng SJ. 2013. Association of specific pectin methyltransferases with Al-induced root elongation inhibition in rice. Physiologia Plantarum 148, 502–511.

Yu P, Wang C, Xu Q, Feng Y, Yuan X, Hu H, Wang Y, Tang S, Wei X. 2011. Detection of copy number variations in rice using array-based comparative genomic hybridization. BMC Genomics 12, 372.

Zhang D, Hua Y, Wang X, Zhao H, Shi L, Xu F. 2014. A high-density genetic map identifies a novel major QTL for boron efficiency in oilseed rape (Brassica napus L.). PLoS ONE 9, e120089.

Zhang XD, Zhao XX, Yang ZM. 2018. Identification of genomic ATP binding cassette (ABC) transporter genes and Cd-responsive ABCs in Brassica napus. Gene 664, 139–151.

Zhao FJ, Hamon RE, Lombi E, McLaughlin MJ, McGrath SP. 2002. Characteristics of cadmium uptake in two contrasting ecotypes of the hyperaccumulator Thlaspi caerulescens. Journal of Experimental Botany 53, 535–543.

Zhao J, Connorton JM, Guo Y, Li X, Shimakawa T, Hirschi KD, Pittman JK. 2009. Functional studies of split Arabidopsis Ca²⁺/H⁺ exchangers. The Journal of Biological Chemistry 284, 34075–34083.

Zhou T, Hua Y, Zhang B, Zhang X, Zhou Y, Shi L, Xu F. 2017. Low-boron tolerance strategies involving pectin-mediated cell wall mechanical properties in Brassica napus. Plant & Cell Physiology 58, 1991–2005.

Zhou ZS, Song JB, Yang ZM. 2012. Genome-wide identification of Brassica napus microRNAs and their targets in response to cadmium. Journal of Experimental Botany 63, 4597–4613.

Zhu CQ, Cao XC, Zhu LF, et al. 2018. Ammonium mitigates Cd toxicity in rice (Orzsa sativa) via putrescine-dependent alterations of cell wall composition. Plant Physiology and Biochemistry 132, 189–201.

Zhu XF, Wang ZW, Wan JX, Wu Y, Wu YR, Li GX, Shen RF, Zheng SJ. 2015. Pectin enhances rice (Orzsa sativa) root phosphorus remobilization. Journal of Experimental Botany 66, 1017–1024.