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## Enhanced expression of radish-specific proteins in a disomic rapeseed-radish chromosome addition line resistant to root-knot nematodes

Nachweis stark exprimierter Rettich-spezifischer Proteine in einer disomen Raps-Rettich-Additionslinie mit Resistenz gegen Wurzelgallennematoden

### Summary

Root-knot nematodes cause significant economic losses worldwide each year. Species with especially high relevance in the tropical and subtropical part of China are *Meloidogyne incognita* and *M. javanica*. The disomic rapeseed-radish chromosome addition line *ee* was shown in former experiments to be highly resistant against these southern root-knot nematodes. In this study, differentially expressed proteins in the roots of the addition line *ee*, its radish donor line and its rapeseed recipient line cv. 'Madora' were screened by two-dimensional gel electrophoresis and Image Master 5.0 software. These proteins were identified by MALDI-TOF-TOF/MS. Three radish-specific proteins with an increased expression in rapeseed-radish addition line *ee* will be further analyzed for their potential role in nematode resistance.

**Key words:** rapeseed, radish, root-knot nematodes, *Meloidogyne incognita*, *Meloidogyne javanica*, resistance

### Zusammenfassung

Wurzelgallennematoden sind weltweit für ganz erhebliche Ertrags- und Qualitätseinbußen verantwortlich. Für den tropischen und subtropischen Teil Chinas sind insbesondere *Meloidogyne incognita* und *M. javanica* von Bedeutung. Die disome Additionslinie *ee* einer komplet-

ten Serie von Raps-Rettich-Additionslinien hatte sich in vorherigen Versuchen als resistent gegenüber diesen beiden Nematodenarten erwiesen. Das Proteinmuster dieser disomen Additionslinie wurde mittels zweidimensionaler Gelelektrophorese und Image Master 5.0 Software mit dem des Rettich-Donors und dem des Raps-Rezipienten verglichen. Differenziell exprimierte Proteine wurden mittels MALDI-TOF-TOF/MS identifiziert. Drei Rettich-spezifische Proteine, die eine erhöhte Expression in der Additionslinie *ee* aufwiesen, sollen in einem zweiten Schritt auf eine potentielle Funktion im Resistenzmechanismus untersucht werden.

**Stichwörter:** Raps, Rettich, Wurzelgallennematoden, *Meloidogyne incognita*, *Meloidogyne javanica*, Resistenz

### Introduction

Root-knot nematodes (RKN) of the genus *Meloidogyne* cause severe damages to a great number of crops worldwide (ELLING, 2013). Infections with these obligate endoparasites result in poor growth and reduced quality. Furthermore resistance against other abiotic and biotic stress factors can be reduced significantly. *M. hapla*, *M. arenaria*, *M. incognita* and *M. javanica* are major pests worldwide. In the southern part of China, especially *M. incognita* and *M. javanica* cause severe problems for various agricultural and horticultural crops (PAN, 1984;

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LI and YU, 1991; WANG et al., 2001). The control of RKNs by application of nematicides to the soil has been more and more restricted because it is not only ineffective but also a severe danger to the environment. The ecologically and economically best alternative would be the improvement of cultivars using resistance genes.

Oil radish (*Raphanus sativus* L.) possesses many favourable agronomic characters for usage in breeding of different *Brassica* species. It shows not only tolerance against abiotic stress factors but is also highly resistant against different pathogens. Especially interesting for introgression into *Brassica* species would be the resistances against clubroot, fusarium wilt, downy mildew, black rot, some viruses and nematodes. To transfer valuable traits with agronomic value from *R. sativus* to *B. napus*, a complete set of nine monosomic rapeseed-radish chromosome addition lines (*a* to *i*) was developed (PETERKA et al., 2004) and converted to disomic addition lines *aa* to *ii* (BUDAHN et al., 2008). This complete set of disomic addition lines can be used to assign resistance traits to individual radish chromosomes. Usefulness was first demonstrated by assigning the radish resistance gene *Hs1<sup>Rph</sup>*, which is effective against beet cyst nematode *Heterodera schachtii*, to radish chromosome *d* (BUDAHN et al., 2009). ZHANG et al. (2014) used the same set to test the resistance behavior against *M. incognita* and *M. javanica*. The disomic addition line *ee* was shown to be highly resistant against both southern root-knot nematodes.

Proteomics is a cutting-edge approach for revealing dynamic changes of protein patterns in response to different abiotic and biotic stresses. A two-dimensional gel electrophoresis (2D-GE) is a classical tool routinely used to understand diverse plant stress response mechanisms. So it has been applied to study *R. sativus* and *B. napus* reactions to salinity, drought and high oxidative stress (KOH et al., 2015; WANG et al., 2016; SUN et al., 2017). However, only a few proteomic analyses were conducted to analyze plant reaction to the stress caused by nematode infection (VILLETH et al., 2015; AL-IDRUS et al., 2017). Aim of this experiment was to identify radish-specific proteins highly expressed in a rapeseed-radish addition line *ee* resistant against *M. incognita* and *M. javanica*.

## Materials and methods

### Plant materials and nematode treatments

Three lines were analyzed: 1) the disomic rapeseed-radish chromosome addition line *ee*, resistant to the root-knot nematodes *M. incognita* and *M. javanica*; 2) the recipient *Brassica napus* cv. 'Madora' and 3) the donor of chromosome *e*, root-knot nematode resistant oil radish strain A24.

The seeds were sown into a nursery plate. After one month, the young plants were transplanted into plastic pots (9 cm×9 cm) with 400 g soil with root-knot nematode population from Jiashui in China. Morphological investigations (ZHANG et al., 2014) showed that the soil

contained predominantly larvae and egg masses of *M. incognita* (55%) and *M. javanica* (34%) and a small percentage (11%) of *M. arenaria*. The plants were cultivated in a climatic chamber under controlled conditions at 18°C for 16 h/13°C for 8 h day-night for two months. Afterwards the roots were stored at -80°C for further analysis.

### Protein isolation and 2D gel electrophoresis

Total proteins were extracted from 3.0 g root material (sampled from four plants) of addition line *ee*, A24 and rapeseed cv. 'Madora', respectively. Roots were used for protein extraction with TCA (Trichloroacetic acid)/acetone, as previously described (YU et al., 2015). Protein concentration was determined using a standard method (BRADFORD, 1976). Briefly, 600 µg total protein was dissolved in 500 µL of rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]propanes), 65 mM DTT (Dithiothreitol), 0.2% IPG (Immobilized pH gradient) buffer, 0.001% bromophenol blue) and loaded on a IPG strip. IPG strips possessed a pH range from 4.0 to 7.0 in order to obtain good resolution and reproducibility. Isoelectric focusing was conducted in IPGphor II under following conditions: 250 V for 30 min, 500 V for 30 min, 1,000 V for 30 min, 8,000 V for 4 h, followed by 8,000 V for 65,000 Vh. The strips were sequentially placed in equilibration buffer-1 (6 M urea, 2% SDS, 50 mM Tris-HCl pH 8.8, 20% glycerol, 2% DTT) and equilibration buffer-2 (6 M urea, 2% SDS, 50 mM Tris-HCl pH 8.8, 20% glycerol, 2.5% iodoacetamide) for 20 min, respectively. The equilibrated strips were run in GE Ettan DAL Tsix (GE, USA) using 12.5% SDS-PAGE gels. The gels were stained by Coomassie Brilliant Blue G-250, scanned by a UMAX Power Look scanner at a resolution of 300 dpi gray scale mode and then analyzed with Image Master 2D Platinum software version 5.0 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The parameters were set to saliency 2.0 and minimum area to 5.0. Clearly separated protein spots with a twofold or higher expression difference between different samples were selected and analyzed by mass spectrometry.

### Protein identification

Protein spots underwent processes of bleaching, trypsin enzymolysis, extraction and ziptip desalination and then the samples were mixed with 5 mg/mL *o*-cyano-4-hydroxycinnamic acid at a ratio of 1:1 (v/v). They were analyzed by 4800 Proteomics Analyzer MALDI-TOF-TOF/MS (Matrix-assisted laser desorption ionization – time of flight – time of flight/mass spectrometry) of the company ABI (Applied Biosystems, USA). The data were obtained in the positive ion mode and automatic mode of data collection. The PMFs (Peptide mass fingerprint) were collected from monoisotopic peaks falling in the *m/z* range of 800–4,000 Da. The data of mass peak obtained by MS were analyzed by the Mascot 2.2 software using the NCBI *Brassicarapa* and *Raphanussativus* protein database as reference.

## Results

### Identification of differentially expressed proteins

To get first indication for proteins potentially involved in the nematode resistance mechanism, 2D-GE was carried out for rapeseed-radish addition line *ee*, the corresponding recipient cv. 'Madora' and the oil radish chromosome donor A24 to determine the differently expressed proteins in root extracts. IPG strips possessing a pH range of 4–7 were chosen to separate the total root proteins. The 2D-GE gels were analyzed by Image Master 5.0. The Figure 1 shows the 736, 1184 and 1163 protein spots detected in the root extracts of A24, 'Madora' and addition line *ee*, respectively. In comparison to the protein pattern of rapeseed cv. 'Madora', for the rapeseed-radish addition line *ee*. Twenty four protein spots were identified, showing at least twofold change in intensity. Fifteen of these

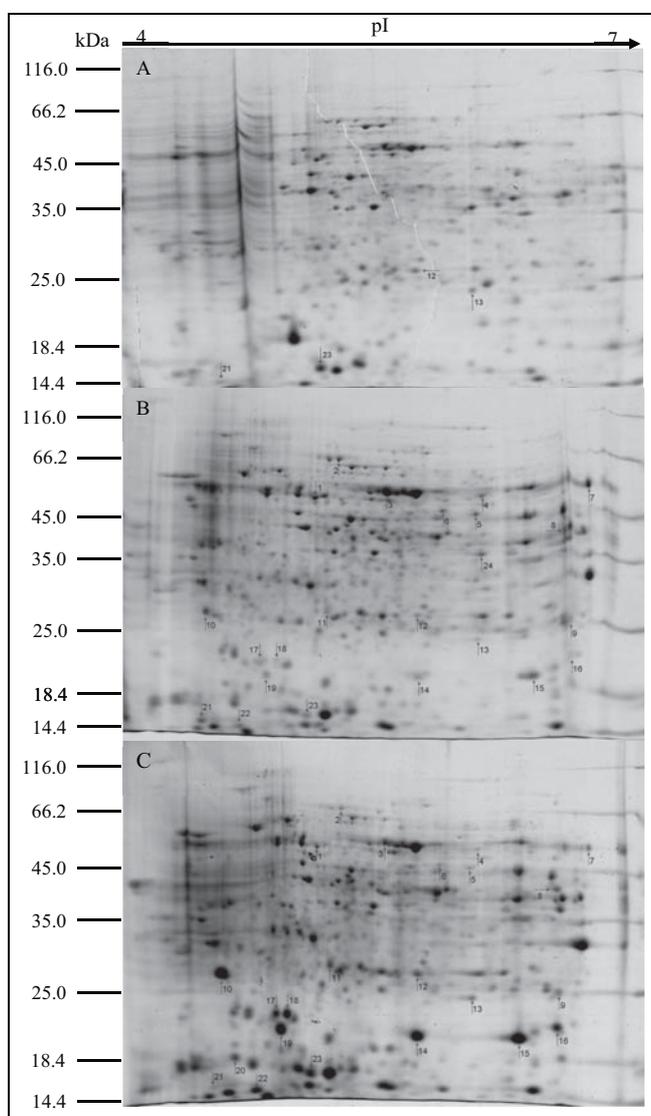
24 proteins were up-regulated and 9 were down-regulated in line *ee*. Twenty-one of them were identified successfully by the MALDI-TOF-TOF/MS analysis (Table 1). Three of the 15 up-regulated proteins were shown to be radish-specific or higher expressed in oil radish A24 than in rapeseed cv. 'Madora': the nucleoside diphosphate kinase 1-like isoform X1 (spot 12), kunitz-type serine protease inhibitor DrTI-like (spot 13) and endogenous alpha-amylase/subtilisin inhibitor-like (spot 23). In addition, the trypsin inhibitor BvTI-like (spot 21) was down-regulated in the addition line *ee* and in oil radish A24.

## Discussion

The complete set of disomic rapeseed-radish addition lines allows to study the effect of individual radish chromosomes on resistance behavior. ZHANG et al. (2014) evidenced that resistance against *M. incognita* and *M. javanica* is caused by one gene or some closely linked resistance genes. In contrast to the results for *M. hapla* where a quantitative resistance was found on radish chromosome *a* (PETERKA et al., 2010), a qualitative resistance against the southern root-knot nematodes *M. incognita* and *M. javanica* was detected on radish chromosome *e*. SHIRASAWA and KITASHIBA (2017) proposed recently a new harmonized nomenclature of the radish chromosomes. According to this nomenclature the chromosome *e* will be named as Rs5.

To identify proteins potentially involved in the resistance mediated by radish chromosome *ee*, the protein pattern of the nematode resistant rapeseed-radish addition line was compared to the rapeseed cv. 'Madora' recipient. Fifteen proteins were up-regulated. The most up-regulated protein is spot 18 with a ratio of 14.02 between addition line *ee* and rapeseed cv. 'Madora'. Among the up-regulated proteins, four protein spots were identified as kunitz-type serine protease inhibitor DrTI-like protein, three as endogenous alpha-amylase/subtilisin inhibitor-like protein. Representatives of both groups were also found to be highly expressed in oil radish strain A24 (spots 12, 13 and 23: Fig. 1A). Additionally, spot 21 was reduced in intensity for both, the addition line *ee* and A24.

Nucleoside diphosphate kinase (NDPK) is mainly involved in the balance of nucleoside diphosphate and nucleoside triphosphates. NDPK plays an important role in plant growth and development (PAN et al., 2000), reaction on clubroot infection (CAO et al., 2008), abiotic stress (CHEN et al., 2012; FATEHI et al., 2012), hormone response (FATEHI et al., 2012) and DNA replication (KOPYLOV et al., 2015). Recent research has shown that potatoes overexpressing *StNDPK1* exhibit a greater root length (DORION et al., 2017). *AtNDPK1* regulates stress through the MAPK (Mitogen-activated protein kinase) signaling pathway (OVEČKA et al., 2014). When subjected to mechanical damage, the expression of *SINDPK1* was up-regulated in tomato seedlings, stems and leaves (HARRIS et al., 1994). *Arabidopsis* overexpressing *OsNDPK1* was shown to up-regulate the resistance genes *PR1* and



**Fig. 1.** The 2D-GE protein maps of root samples. A) oil radish A24; B) rapeseed cv. 'Madora'; C) disomic rapeseed-radish addition line *ee*. The pI gradient is shown at the top of gels, and sizes of molecular mass markers are shown on the left side.

**Table 1. Differently expressed proteins identified by MALDI-TOF-TOF/MS in the roots of rapeseed-radish chromosome addition line eecompared to the recipient rapeseed cv. 'Madora' roots**

Spot	Locus No.	Protein	Mr/pI	Pep. Count	Score	Ratioee/Madora
<b>Metabolism</b>						
3	XP_013673398.1	PREDICTED: ATP synthase subunit beta-2, mitochondrial	53.8/5.39	29	689	0.48
4	XP_013723181.1	PREDICTED: 3-isopropylmalate dehydratase large subunit-like	54.79/7.12	15	162	0.48
24	XP_013748848.1	PREDICTED: malate dehydrogenase 1, mitochondrial-like	35.9/8.23	13	323	sp
<b>Stress and Defense</b>						
1	XP_013640865.1	PREDICTED: tubulin alpha-6 chain	49.51/4.93	18	330	0.30
5	XP_013748399.1	PREDICTED: S-adenosylmethionine synthase 3	42.47/5.76	14	193	0.49
6	XP_013722790.1	PREDICTED: S-adenosylmethionine synthase isoform X2	43.16/5.67	20	254	0.48
7	XP_013650116.1	PREDICTED: epidermis-specific secreted glycoprotein EP1-like	49.28/6.88	19	178	0.49
9	XP_013710446.1	PREDICTED: germin-like protein subfamily 1 member 8	23.25/6.81	6	71	3.86
12	XP_013649391.1	PREDICTED: nucleoside diphosphate kinase 1-like isoform X1	16.38/6.29	6	248	2.01
13	XP_013698983.1	PREDICTED: kunitz-type serine protease inhibitor DrTI-like	23.33/5.54	8	192	6.34
14	XP_013698983.1	PREDICTED: kunitz-type serine protease inhibitor DrTI-like	23.33/5.54	7	266	3.41
15	XP_013698983.1	PREDICTED: kunitz-type serine protease inhibitor DrTI-like	23.33/5.54	8	248	2.03
16	XP_013727053.1	PREDICTED: kunitz-type serine protease inhibitor DrTI-like	23.31/5.12	3	121	13.52
20	XP_013701228.1	PREDICTED: endogenous alpha-amylase/subtilisin inhibitor-like	23.87/5.38	6	141	sp
21	XP_013699343.1	PREDICTED: trypsin inhibitor BvTI-like	22.56/4.96	10	235	0.36
22	XP_013701228.1	PREDICTED: endogenous alpha-amylase/subtilisin inhibitor-like	23.87/5.38	5	136	2.11
23	XP_013701228.1	PREDICTED: endogenous alpha-amylase/subtilisin inhibitor-like	23.87/5.38	6	182	6.10
<b>Transport</b>						
2	XP_013676805.1	PREDICTED: V-type proton ATPase catalytic subunit A-like	68.61/5.11	26	274	0.29
<b>Unknown</b>						
11	CDY17863.1	BnaC04g03560D	44.63/8.05	12	334	2.17
17	CDX72973.1	BnaC06g34210D	23.24/4.94	7	282	2.57
18	CDX72973.1	BnaC06g34210D	23.24/4.94	7	502	14.02

Mr: relative molecular mass  
 pI: isoelectric point  
 sp: specific for addition line ee

*NPR1* relative to wild-type. Thus, *NDPK1* is probably involved in plant defense mechanisms.

Serine protease inhibitors (Serpins) are the most diverse protease inhibitor superfamily. There are 18

genes in the *Arabidopsis* genome. SerpinZx from *Hordeum vulgare* L. inhibits trypsin, chymotrypsin, and cofactors in blood clotting (DAHL et al., 1996). The lack of serine proteases such as chymotrypsin in plants suggests

that the serpin in plant is likely to be a protease that inhibits plant pathogens.

Trypsin inhibitors are a class of small peptide molecules that inhibit the activity of trypsin hydrolysis and are commonly found in plant storage organs such as seeds, roots and tubers. Transgenic plants including dozens of different types of trypsin inhibitors have shown strong resistance against different pests. Its principle of action is to inhibit the activity of insects digestive enzymes to prevent the effective utilization of food proteins by pests and to reduce the food intake of insects, followed by a stop of development and the death of insects due to the lack of necessary nutrients. Nevertheless feeding of nematodes is completely different from that of insects.

Our results show that *NDPK* and serpins were highly expressed in resistant oil radish strain A24 and addition line *ee*. To check if the candidate genes are really involved in the resistance mechanism, in the next experimental series their expression level should be measured more in detail in the time frame from nematode infection to the development of egg masses. In parallel, the development of the feeding sites should be investigated by cytological studies.

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