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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

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;
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 (Petrerka 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 Hs1ph, 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., 2013; 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 (Vileth et al., 2017; 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 Jianshui 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)dimethyl-ammonio]propanes), 65 mM DTT (Diithiothreitol), 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 Tiix (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 a-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 Brassinicapa 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 isof orm X1 (spot 12), kunitz-type serine protease inhibitor DrTI-like (spot 13) and endogenous alpha-amy lase/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 Kitashita (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-amy lase/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 (Pang 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 (Koplov et al., 2015). Recent research has shown that potatoes overexpressing StNDPK1 exhibit a greater root length (Dorig et al., 2017). AtNDPK1 regulates stress signaling through the MAPK (Mitogen-activated protein kinase) signaling pathway (Ovecˇka 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 PRI and...
Table 1. Differently expressed proteins identified by MALDI-TOF-TOF/MS in the roots of rapeseed-radish chromosome addition line compared to the recipient rapeseed cv. ‘Madora’ roots

| Spot | Locus No. | Protein                                                                 | Mr/pl | Pep. Count | Score | Ratio ee/Madora |
|------|-----------|-------------------------------------------------------------------------|-------|------------|-------|-----------------|
| 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              |

Metabolism

| Spot | Locus No. | Protein                                                                 | Mr/pl | Pep. Count | Score | Ratio ee/Madora |
|------|-----------|-------------------------------------------------------------------------|-------|------------|-------|-----------------|
| 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 isofrom 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 isofrom 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-amylose/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-amylose/subtilisin inhibitor-like          | 23.87/5.38 | 5          | 136   | 2.11            |
| 23   | XP_013701228.1 | PREDICTED: endogenous alpha-amylose/subtilisin inhibitor-like          | 23.87/5.38 | 6          | 182   | 6.10            |

Stress and Defense

Transport

| Spot | Locus No. | Protein                                                                 | Mr/pl | Pep. Count | Score | Ratio ee/Madora |
|------|-----------|-------------------------------------------------------------------------|-------|------------|-------|-----------------|
| 2    | XP_013676805.1 | PREDICTED: V-type proton ATPase catalytic subunit A-like              | 68.61/5.11 | 26         | 274   | 0.29            |

Unknown

| Spot | Locus No. | Protein                                                                 | Mr/pl | Pep. Count | Score | Ratio ee/Madora |
|------|-----------|-------------------------------------------------------------------------|-------|------------|-------|-----------------|
| 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. Nethertheless 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.

Acknowledgements

This work was supported by Natural Science Foundation of China (31460470), Yunnan Natural Science Foundation (2015FD059) and Sino-Germany Cooperation on Agricultural Science and Technology (2017/2018 project 37/12–13).

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