Sensitivity and Speed of Induced Defense of Cabbage (*Brassica oleracea* L.): Dynamics of *BoLOX* Expression Patterns During Insect and Pathogen Attack

Si-Jun Zheng,¹ Jeroen P. van Dijk,² Maaike Bruinsma,¹ and Marcel Dicke¹

¹Laboratory of Entomology, Wageningen University, P.O. Box 8031, 6700 EH Wageningen, The Netherlands; ²RIKILT-Institute of Food Safety, Wageningen University and Research Centre, P.O. Box 230, 6700 AE Wageningen, The Netherlands

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The lipoxygenase pathway is involved in the early steps of plant responses to herbivorous insects and phytopathogens. Induced defenses in the crucifer *Brassica oleracea* have been well documented. Here, we have cloned a *LIPOXYGENASE* (*LOX*) from *B. oleracea* (*BoLOX*). The sequence reveals that the *BoLOX* protein has a transit peptide for chloroplast targeting, which is characteristic for class 2 *LOXs* involved in jasmonic acid (JA) biosynthesis which takes place in the chloroplast. Phylogenetic analysis shows that *BoLOX* is closely related to *B. napus* *BnLOX2fl* and *Arabidopsis thaliana* *AtLOX2*, which mediates JA biosynthesis. *BoLOX* also shares functional characteristics with *AtLOX2*; *BoLOX* is inducible by wounding, JA treatment, and herbivores such as caterpillars (*Pieris rapae*, *P. brassicae*, and *Mamestra brassicae*), spider mites (*Tetranychus urticae*), locusts (*Schistocerca gregaria*), and a bacterial pathogen (*Pseudomonas syringae* pv. *tomato*). Of these, *Pieris* spp. caterpillars also induce *AtLOX2* and JA biosynthesis in *Arabidopsis*. However, the aphid *Myzus persicae* did not induce *BoLOX*, which agrees with previous reports that this aphid induces neither *AtLOX2* nor JA biosynthesis in *Arabidopsis*. Quantitative expression analysis of temporal, spatial, and density-dependent *BoLOX* transcript levels through real-time quantitative polymerase chain reaction demonstrated that *BoLOX* is maximally expressed after feeding by only two first-instar caterpillars for 24 h. Systemic expression was approximately 10-fold lower than local expression for herbivore-induced responses. The good correlation of *BoLOX* transcript levels with reports in the literature on induced defenses of *B. oleracea* is discussed.

Plants serve as food to a wide variety of consumers. The most diverse and abundant group of herbivores consists of insects. In all, 45% of the approximately 1 million described insect species feed on plants (Schoonhoven et al. 2005). Plants have evolved a range of defenses to ward off this diversity of attackers (Kessler and Baldwin 2002; Schoonhoven et al. 2005; Walling 2000). These defenses can be classified as constitutive defenses that are present independent of attack and

induced defenses that are induced by attack (Dicke et al. 2003; Karban and Baldwin 1997).

Induced defenses are mediated by signal transduction that links the damage with the changed phenotype. There are three main signal-transduction pathways that underlie induced defenses (i.e., the jasmonate, shikimate, and ethylene pathways) (Dicke and Van Poecke 2002; Kessler and Baldwin 2002). Of these pathways, the jasmonate pathway seems to play a dominant role in insect-induced, mite-induced, and wound-induced plant responses. For instance, jasmonates have been shown to be induced by *Manduca sexta* caterpillars in native tobacco plants (Kahl et al. 2000) or by *Pieris rapae* caterpillars in *Arabidopsis* (Reymond et al. 2004). A key enzyme in the biosynthetic pathway toward jasmonic acid (JA) is lipoxygenase (*LOX*) that uses linolenic acid as a substrate. The *LOX* gene has been shown to be induced in response to wounding or herbivory in plants such as *Arabidopsis*, native tobacco, and tomato (Bell et al. 1995; Halitschke and Baldwin 2003; Heitz et al. 1997). Disrupting *LOX* RNA accumulation results in a plant phenotype that is impaired in defense against herbivorous insects such as caterpillars (Kessler et al. 2004; Royo et al. 1999). *AtLOX2* is induced by insect herbivory and *Atlox2* antisense plants are impaired in anti-insect defense (Bell et al. 1995; Van Poecke and Dicke 2002; Van Poecke et al. 2001).

After major developments in the knowledge of signal-transduction in induced defenses of *Arabidopsis* against pathogens (Pieterse and Van Loon 1999; Ryals et al. 1995), this model plant also has been incorporated in studies on induced plant defenses against herbivorous insects (Barth and Jander 2006; Jander et al. 2001; Reymond et al. 2000, 2004; Stotz et al. 2002; Van Poecke and Dicke, 2002, 2004; Wittstock et al. 2004). *Arabidopsis* provides a valuable model plant for ecologists to use to investigate mechanisms of induced defense through a molecular genetic approach because full-genome microarrays, extensive mutant collections, and ample information on signal transduction are available (Mauricio 1998; Mauricio and Rausher 1997; Mauricio et al. 1997; Mitchell-Olds 2001; Van Poecke and Dicke 2004). However, to investigate the effects of genes on community ecology, other crucifers are more suitable because, in nature, *Arabidopsis* has little interaction with herbivorous insects because they are active in a different time window. There is extensive information on the ecology of brassicaceous plants (Agrawal 2000; Gols et al. 2005; Harvey et al. 2003; Louda and Rodman 1996; Nielsen and de Jong 2005; Root 1973; Shiojiri et al. 2002; van Dam et al. 2004). *Brassica* spp. are attacked by species from very dif-

Corresponding author: Marcel Dicke; Telephone: +31-317-484311; Fax: +31-317-484821; E-mail: marcel.dicke@wur.nl

Nucleotide sequence data for the *BoLOX* and *GAPDH* genes are available in the GenBank database under gene accession numbers EF123056 and EF123055, respectively.

ferent insect orders, including beetles, caterpillars, aphids, and thrips, and are pollinated by hymenopteran insects. The herbivorous insects are attacked by a range of carnivorous insects, including parasitic wasps and predatory insects. Characteristics of *Brassica* plants have been documented to affect insects up until the fourth trophic level (Harvey et al. 2003); consequently, the phenotype of *Brassica* plants can have profound effects on community processes. *Brassica oleracea* is commonly known as a cultivated plant, and agricultural varieties have been investigated intensively for induced defenses against insects (Mattiacci et al. 1995; Reymond et al. 2004; Shiojiri et al. 2002; Vuorinen et al. 2004). Yet, wild populations of this plant exist in Europe and provide interesting potential for ecological investigations (Moyes and Raybould 2001; Moyes et al. 2000).

At present, there are interesting options for using *Arabidopsis* as a stepping stone toward *Brassica* spp. in a molecular ecological research program. For instance, 70-mer oligomicroarrays based on the *Arabidopsis* genome can be used for transcriptomics in *Brassica* spp. and *Arabidopsis* sequence information can be used to clone related genes in other brassicaceous plants (Lee et al. 2004). Ecologists are interested in specificity of plant–attacker interactions and in quantitative data on temporal variation in responses of plants to their attackers. So far, transcriptomic analyses of plant–insect interactions have concentrated mostly on elucidating which genes are upregulated, but quantitative analyses have remained rare (Halitschke and Baldwin 2003; Park et al. 2002). However, the development of quantitative real-time polymerase chain reaction (PCR) allows careful quantitative analysis of gene expression patterns (Halitschke and Baldwin 2003; Halitschke et al. 2004). Such information will provide important new details on the temporal patterns of gene expression, and comparing these patterns among responses to different attackers will provide information on responses of plants to different community members. This is the topic of the present study.

We have cloned a *B. oleracea* LOX gene that is inducible by wounding and insect herbivory. Through quantitative real-time PCR, we have investigated its transcript levels in response to feeding by four insect species of different orders, a mite species, mechanical wounding, JA application, and a pathogenic microorganism. Herbivore-density-related responses as well as temporal patterns in *BoLOX* transcript levels have been quantified for damaged as well as systemic leaves. The data show that transcript levels of this *BoLOX* gene are strongly upregulated in response to feeding damage by locusts, spider mites, and three species of caterpillars, including one generalist and two specialists, and in response to infection with the pathogenic bacterium *Pseudomonas syringae*. Maximum transcript levels (up to 10,000-fold compared with uninfested control leaves) in response to caterpillar feeding were reached after 24 h and, surprisingly, the maximum transcript level was little affected by the number of caterpillars that fed on the plant. Through quantitative real-time PCR, *BoLOX* RNAs already were detected 1 h after caterpillar feeding. This contrasted with the time (24 h) when *BoLOX* RNAs were first detected when conventional RT (reverse-transcription) PCR was used to record RNA accumulation. Mechanical damage and JA also induced accumulation of *BoLOX* RNA. In contrast to the relationship between herbivore density and *BoLOX* transcript levels, there was a linear relationship between JA dose and *BoLOX* transcript levels.

RESULTS

Isolation and characterization of *BoLOX* cDNA.

A partial sequence (1,021 bp) of the cabbage LOX gene was obtained after sequencing fragments obtained by RT-PCR using

degenerate oligonucleotide primers. A full-length cDNA of 2,785 bp was isolated using rapid amplification of cDNA ends (5'-RACE and 3'-RACE). This cDNA clone contained an open reading frame starting with an ATG codon preceded by an in-frame stop codon, indicating that it contains the complete coding region. The open reading frame of 2,676 nucleotides encoded a predicted protein of 891 amino acids. This cDNA clone was designated *B. oleracea* LOX gene (*BoLOX*). The deduced protein sequence of *BoLOX* showed high variability in the N-terminus and high similarity with other known LOX proteins in the C-terminus (Fig. 1).

The iPSORT protein-sorting program predicted subcellular localization of *BoLOX* in the cytoplasm and chloroplast because *BoLOX* contains a transit peptide for chloroplast and mitochondrial targeting. Similar to *AtLOX2* (Bell and Mullet 1993), potato H1 (LOX2) and H3 (LOX3) (Royo et al. 1996), and tomato TomLOXC and TomLOXD (Heitz et al. 1997), the *BoLOX* sequence exhibits N-terminal extensions of approximately 60 residues, showing a chloroplast and mitochondrial transit peptide. The general features of transit peptides for chloroplast targeting include a high proportion of hydrophilic and small hydrophobic amino acid residues and a near lack of acidic residues. The *Arabidopsis* and tomato LOX enzymes have been demonstrated to be actively imported into chloroplasts (Bell et al. 1995; Heitz et al. 1997); also, potato H1 (LOX2) and H3 (LOX3) are localized within the chloroplasts (Farmaki et al. 2007). An important function of the chloroplast-localized class 2 LOXs is their involvement in catalyzing the oxygenation of free linolenic acid as the initial step in JA biosynthesis (Wasternack et al. 2006). Like other known plant LOX sequences (Peng et al. 1994), the *BoLOX* sequence exhibits the conserved amino acid sequence HAAVNFGQY present in the C-terminal part (Fig. 1). From the phylogenetic analysis (Fig. 2), it is clear that the nucleotide sequence of *BoLOX* is very close to that from *B. napus* *BnLOX2fl* (Terp et al. 2006) and *Arabidopsis thaliana* *AtLOX2* (Bell and Mullet 1993). *BoLOX* showed 87 and 75% amino acid sequence identity with *B. napus* *BnLOX2fl* and *A. thaliana* *AtLOX2*, respectively. Based on sequence identity with known 13-LOXs, the presence of LOX consensus motifs, and the presence of a transit peptide, it is likely that *BoLOX* encodes a 13-LOX and not a 9-LOX.

Accumulation of *BoLOX* RNA in response to treatment with the phytohormone JA.

The levels of *BoLOX* transcripts in response to treatment with different doses of JA were investigated with RT-PCR, both conventionally and with real-time PCR. RNA was isolated from the leaves of three independent biological replicates, each consisting of a single plant. The transcript levels of *BoLOX* were normalized for transcript levels of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Both conventional and real-time PCR showed an increase of *BoLOX* transcript levels in a JA-concentration-dependent fashion for all three experiments (Fig. 3). With increasing JA concentration from 0, 10, 100, to 1,000 μ M, the transcript level of *BoLOX* significantly increased 24 h after spraying. We chose this time point because *B. oleracea* plants are most attractive to *Cotesia glomerata* wasps at 24 h after treatment with 1 mM JA compared with 1, 2, 3, 4, 6, 48, 72, and 120 h (M. Bruinsma, J. J. van Loon, and M. Dicke, unpublished data). Moreover, the two butterfly species *Pieris rapae* and *P. brassicae* laid fewer eggs on leaves of JA-treated plants after 24 h compared with control plants (Bruinsma et al. 2007). At this time point, maximum *BoLOX* transcript levels were recorded in response to herbivory (discussed below). Plants showed no visual symptoms after application of any of the JA doses (data not shown). The

GAPDH gene that was used as an internal reference showed a stable transcript level among different treatments (data not shown). Relative *BoLOX* transcript levels were 46-, 2,185-, and 14,230-fold, on average, after 10-, 100- and 1,000- μ M JA exposure, respectively, compared with control plants (Fig. 3).

Dynamics of *BoLOX* RNA levels in response to feeding by different herbivores.

Schistocerca gregaria locusts. A single first-instar locust had been placed on one of the youngest fully expanded leaves of each cabbage plant in order to use leaves of similar physiological state among different plants (Fig. 4B). The infested and damaged leaf was denoted as local leaf, whereas one undamaged

leaf directly below the infested leaf was denoted as systemic leaf. The *BoLOX* transcript level was significantly increased 3 days later, both locally in the damaged leaf and in a systemic undamaged leaf. Real time RT-PCR showed 870, and 374-fold increase in *BoLOX* transcript levels in the local and systemic leaves, respectively, compared with control plants (Fig. 5).

Pieris and *Mamestra caterpillars*. Both the effect of caterpillar density and time of feeding on *BoLOX* transcript accumulation were investigated after infestation by caterpillars from the crucifer specialists *P. rapae* and *P. brassicae* (Fig. 4C and D) in a series of experiments. *BoLOX* RNA was visible by conventional RT-PCR after 24 h of feeding by one *P. brassicae* or *P. rapae* larva in the local (infested) leaf (Fig. 6A). *BoLOX*

Fig. 1. Clustal X (1.83) multiple alignment of deduced amino acid sequence of *Brassica oleracea* lipoxygenase (BoLOX) with other selected LOXs. The consensus amino acid sequence of nearly all known plant LOX sequences HAAVNFQY is underlined. Accessions for LOX amino sequences represented in this alignment are *Arabidopsis thaliana* AtLOX1 (NM 104376); *A. thaliana* AtLOX2 (L23968); *A. thaliana* AtLOX3 (AJ249794); *A. thaliana* AtLOX6 (AJ748537); *Solanum tuberosum* potato H1 (X96405); *S. tuberosum* potato H3 (X96406); *Nicotiana attenuata* NaLOX3 (AY254349); *Lycopersicon esculentum* TomLOXC (U37839); *L. esculentum* TomLOXD (U37840); *Glycine max* SoyLOX3 (X13302); *B. napus* BnLOX1f1 (AY162142); and *BnLOX2 f1* (AY162143). Asterisks (*) indicates identical amino acid residues, while colons (:) indicate low and high homologous amino acid residues, respectively.

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RNA accumulation was not detected in control plants (data not shown). The *BoLOX* transcript levels were much higher in both local and systemic leaves after 24 h of feeding by 1 to 32 *P. rapae* larvae compared with *P. brassicae* (Fig. 6A). With increasing numbers of *P. rapae* larvae (from 1 to 2, 4, 8, 16, and 32), the relative *BoLOX* transcript level in the local leaf was significantly increased 894-, 2,769-, 2,961-, 5,663-, 8,980-, and 9,796-fold compared with the undamaged control (Fig. 6B). When the larval density was increased from 2 to 32 caterpillars, *BoLOX* transcript levels in the local leaf were similar ($P > 0.05$). In the systemic (noninfested) leaf, there was a significant increase in *BoLOX* transcript level as well, albeit much lower. The relative *BoLOX* transcript level in the systemic leaf was increased 45-, 169-, 54-, 274-, 265-, and 986-fold after feeding by 1, 2, 4, 8, 16, or 32 larvae, respectively, compared with an undamaged control plant (Fig. 6B). *BoLOX* transcript levels in the systemic leaf were independent of caterpillar density (from 1 to 32; $P > 0.05$).

Because feeding for 24 h by only one first-instar *P. brassicae* or *P. rapae* larva was sufficient to induce *BoLOX* transcripts (Fig. 6A), we investigated the temporal pattern in *BoLOX* transcripts in response to feeding by one first-instar larva for both *P. rapae* and *P. brassicae*. As assessed by conventional RT-PCR, accumulation of *BoLOX* RNA could be visualized only after at least 24 h of caterpillar feeding, in both the local and systemic leaves (data not shown). There was no *BoLOX* RNA accumulation detected in control plants (data not shown). Relative *BoLOX* transcript levels were significantly increased 9-, 25-, 1,274-, 1,488-, 1,658- and 1,930-fold in the local leaf after feeding by one first-instar *P. brassicae* caterpillar for 1 to 96 h (Fig. 7). In the systemic leaf, the induction was much lower than in the local leaf; the relative *BoLOX* transcript level in the systemic leaf was increased only 5-, 5-, 81-, 120-, 165-, and 193-fold compared with the undamaged control plants (Fig. 7). In the systemic leaves, *BoLOX* transcripts were induced significantly only after 24 h and longer. *BoLOX* transcript levels were similar after cat-

TomLOXC	VLSDHSQYHQLVSHWLRTHCCTEPIIATNRQLSAMHPYIRLLHPHFRTMEINALAREA	TomLOXD	AFYKFSAEIGRIEKEIDERNADTNLKNRCGAGVLPYELLAPSSGPGVTCRGVPSVSI
PotatoH3	VCANDAGVHQLVNHWRTHASLEPFLAAHRQLSAMHPYIKLLDPHMYRTLEINGLARQS	NaLOX3	AFYDFSSSEIRRIEKEIDERNADTNLKNRCGAGVLPYELLAPSSGPGVTCRGVPSVSI
TomLOXD	VCANDAGVHQLVNHWRTHASLEPFLAAHRQLSAMHPYIKLLDPHMYRTLEINGLARQS	AtLOX3	AFYGFAAEIGRIEKEIEKRNADPNRRNCAGVLPYELLVPSSEPGVTGCGVPSVSI
NaLOX3	VCSNDAGVHQLVNHWRTHASLEPFLAAHRQLSAMHPYIKLLDPHMYRTLEINALARQS	AtLOX6	YFNKFSEELVKIEKTINERNDKDKLNRTGAGMPPEYELLPTSPHGVTRGIPNSI
AtLOX3	VCSNDAGVHQLVNHWRTHASLEPFLAAHRQLSAMHPYIKLLDPHMYRTLEINALARQS	BnLOX1 f1	AFKFGKDKVKIEKTINERNDENLNRTGAGMPPEYELLPTSPHGVTRGIPNSI
AtLOX6	VCSNDAGVHQLVNHWRTHASLEPFLAAHRQLSAMHPYIKLLDPHMYRTLEINARARQS	AtLOX1	AFKFGKDKVKIEKTINERNDENLNRTGAGMPPEYELLPTSPHGVTRGIPNSI
BnLOX1 f1	VGNDSGNHQLISHWLQTHASIEPFIATNRQPSVLHPVFKLLPHYRDTMNLINARARQS	SoyLOX3	AFKFRGNKLAQIENKLSERNDEKLNRNCGVPMPTYLLPSKEGLTFRGIPNSI
AtLOX1	VGNDSGNHQLISHWLQTHASIEPFIATNRQPSVLHPVFKLLPHYRDTMNLINARARQS		
SoyLOX3	VVNDSCYHQLVSHWLTHAVVEPFIATNRHLSVHPVFKLLPHYRDTMNLINARARQS		
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BoLOX	LVNAGGIIETCFWPGKYSLELSSDVYAKLWRFDEGLPADLISRGGLADETAEHGLRLT		
BnLOX2 f1	LINEGGIIETCFWPGKYSLELSSDVYAKLWRFDEGLPADLISRGGLADETAEHGLRLT		
AtLOX2	LVNAGGIIETCFWPGKYSLELSSDVYAKLWRFDEGLPADLISRGGLADETAEHGLRLT		
PotatoH1	LINANGVIESFFPGKYAIELSSIAAGAEWRFDQALPNQLISRGGLADETAEHGLRLT		
TomLOXC	LINANGVIESFFPGKYAIELSSIAAGAEWRFDQALPNQLISRGGLADETAEHGLRLT		
PotatoH3	LINADGVIEACFTPGRYCMEISAAAYKN-WRFDLEGLPADLISRGGLADETAEHGLRLT		
TomLOXD	LINADGVIEACFTPGRYCMEISAAAYKN-WRFDLEGLPADLISRGGLADETAEHGLRLT		
NaLOX3	LISADGVIEACFTPGRYCMEISAAAYKN-WRFDLEGLPADLISRGGLADETAEHGLRLT		
AtLOX3	LISADGVIEACFTPGRYCMEISAAAYKN-WRFDLEGLPADLISRGGLADETAEHGLRLT		
AtLOX6	LINGGGIIETCFWPGKYSLELSSDVYAKLWRFDEGLPADLISRGGLADETAEHGLRLT		
BnLOX1 f1	LINGGGIIETCFWPGKYSLELSSDVYAKLWRFDEGLPADLISRGGLADETAEHGLRLT		
AtLOX1	LINGGGIIETCFWPGKYSLELSSDVYAKLWRFDEGLPADLISRGGLADETAEHGLRLT		
SoyLOX3	LVNDGGVIEQTFWGRYSVEMSAVYK-DWVFTDQALPADLISRGGLADETAEHGLRLT		
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BoLOX	IDPYFFANDGLMLDALKKEWITECVKHYYDPAALIMDEELQAWWSEVRNIGHGDKKDEP		
BnLOX2 f1	IDPYFFANDGLMLDALKKEWITECVKHYYDPAALIMDEELQAWWSEVRNIGHGDKKDEP		
AtLOX2	IDPYFFANDGLMLDALKKEWITECVKHYYDPAALIMDEELQAWWSEVRNIGHGDKKDEP		
PotatoH1	IDPYFFANDGLMLDALKKEWITECVKHYYDPAALIMDEELQAWWSEVRNIGHGDKKDEP		
TomLOXC	IDPYFFANDGLMLDALKKEWITECVKHYYDPAALIMDEELQAWWSEVRNIGHGDKKDEP		
PotatoH3	IDPYFFANDGLMLDALKKEWITECVKHYYDPAALIMDEELQAWWSEVRNIGHGDKKDEP		
TomLOXD	IDPYFFANDGLMLDALKKEWITECVKHYYDPAALIMDEELQAWWSEVRNIGHGDKKDEP		
NaLOX3	IDPYFFANDGLMLDALKKEWITECVKHYYDPAALIMDEELQAWWSEVRNIGHGDKKDEP		
AtLOX3	IDPYFFANDGLMLDALKKEWITECVKHYYDPAALIMDEELQAWWSEVRNIGHGDKKDEP		
AtLOX6	IDPYFFANDGLMLDALKKEWITECVKHYYDPAALIMDEELQAWWSEVRNIGHGDKKDEP		
BnLOX1 f1	IDPYFFANDGLMLDALKKEWITECVKHYYDPAALIMDEELQAWWSEVRNIGHGDKKDEP		
AtLOX1	IDPYFFANDGLMLDALKKEWITECVKHYYDPAALIMDEELQAWWSEVRNIGHGDKKDEP		
SoyLOX3	IDPYFFANDGLMLDALKKEWITECVKHYYDPAALIMDEELQAWWSEVRNIGHGDKKDEP		
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BoLOX	WWPVLKTQDDLGIVVTIIAWVASGHHAANVFGQYGGYGFNPRFTTIRIMPEEPTDEE		
BnLOX2 f1	WWPVLKTQDDLGIVVTIIAWVASGHHAANVFGQYGGYGFNPRFTTIRIMPEEPTDEE		
AtLOX2	WWPVLKTQDDLGIVVTIIAWVASGHHAANVFGQYGGYGFNPRFTTIRIMPEEPTDEE		
PotatoH1	WWPVLKTQDDLGIVVTIIAWVASGHHAANVFGQYGGYGFNPRFTTIRIMPEEPTDEE		
TomLOXC	WWPVLKTQDDLGIVVTIIAWVASGHHAANVFGQYGGYGFNPRFTTIRIMPEEPTDEE		
PotatoH3	WWPVLKTQDDLGIVVTIIAWVASGHHAANVFGQYGGYGFNPRFTTIRIMPEEPTDEE		
TomLOXD	WWPVLKTQDDLGIVVTIIAWVASGHHAANVFGQYGGYGFNPRFTTIRIMPEEPTDEE		
NaLOX3	WWPVLKTQDDLGIVVTIIAWVASGHHAANVFGQYGGYGFNPRFTTIRIMPEEPTDEE		
AtLOX3	WWPVLKTQDDLGIVVTIIAWVASGHHAANVFGQYGGYGFNPRFTTIRIMPEEPTDEE		
AtLOX6	WWPVLKTQDDLGIVVTIIAWVASGHHAANVFGQYGGYGFNPRFTTIRIMPEEPTDEE		
BnLOX1 f1	WWPVLKTQDDLGIVVTIIAWVASGHHAANVFGQYGGYGFNPRFTTIRIMPEEPTDEE		
AtLOX1	WWPVLKTQDDLGIVVTIIAWVASGHHAANVFGQYGGYGFNPRFTTIRIMPEEPTDEE		
SoyLOX3	WWPVLKTQDDLGIVVTIIAWVASGHHAANVFGQYGGYGFNPRFTTIRIMPEEPTDEE		
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BoLOX	LKEFYEEPEKVLKTFPSKQATQVMVTLDDLSTHSPDEEYLGE--EPEASWVDPVIFA		
BnLOX2 f1	LKEFYEDPEKTMKTFPSKQATQVMVTLDDLSTHSPDEEYLGE--EPEASWAEHPVIFA		
AtLOX2	LKEFYESPEKVLKTFPSKQATQVMVTLDDLSTHSPDEEYLGE--EPEASWAEHPVIFA		
PotatoH1	WEMFNKPEEALRCFSPQIQATKVMATLIVLSNHSDEEYLGE--KIEPYWAEHPVIFA		
TomLOXC	WEMFNKPEEALRCFSPQIQATKVMATLIVLSNHSDEEYLGE--KIEPYWAEHPVIFA		
PotatoH3	YAVFLADPKYFFSALPSLLQATKFMVAVDTLSTHSPDEEYLGE--RHQPSTWGDAAEIV		
TomLOXD	YAVFLADPKYFFSALPSLLQATKFMVAVDTLSTHSPDEEYLGE--RHQPSTWGDAAEIV		
NaLOX3	YAVFHDHPKQYFFSALPSLLQATKFMVAVDTLSTHSPDEEYLGE--RHQPSTWGDAAEIV		
AtLOX3	YASFISHPEKYFFSMPSLAQTKFMVAVDTLSTHSPDEEYLGE--RHQPSTWGDAAEIV		
AtLOX6	YEMFMRNPQYFSLGSLPTQLQATKFMVAVDTLSTHSPDEEYLGE--RHQPSTWGDAAEIV		
BnLOX1 f1	FEELKPNPKVFLKSIQAQLTLLGISLIEILSTHSSDEVYLGQ--RDSKEWAEKEALE		
AtLOX1	FEELKPNPKVFLKSIQAQLTLLGISLIEILSTHSSDEVYLGQ--RDSKEWAEKEALE		
SoyLOX3	YEELKPNPKAYLKTIPTKFTLIDLIVLIEILSRHASDEVYLGQ--RDNPNWTSDEKRALE		
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BoLOX	AYERFKGRLKHLVDIDERNVNVSLKNRAGAGVVKYELLKPISEPQVGMGVPYPSVSI		
BnLOX2 f1	AYERFKGRLKHLVDIDERNVNVSLKNRAGAGVVKYELLKPISEPQVGMGVPYPSVSI		
AtLOX2	AYERFKGRLKHLVDIDERNVNVSLKNRAGAGVVKYELLKPISEPQVGMGVPYPSVSI		
PotatoH1	AFEFVSGKLKELEGIIDARNNDKSLNRRNAGAGVMPYELLKPISEPQVGMGVPYPSVSI		
TomLOXC	AFEFVSGKLKELEGIIDARNNDKSLNRRNAGAGVMPYELLKPISEPQVGMGVPYPSVSI		
PotatoH3	AFYKFSAEIGRIEKEIDERNANTKLNRCGAGVLPYELLAPSSGPGVTCRGVPSVSI		

Fig. 1. Continued from previous page.

erpillar feeding times that ranged from 24 to 96 h in both the local and systemic leaves. We also investigated the *BoLOX* transcript pattern after feeding by one first-instar larva of the generalist caterpillar *Mamestra brassicae* in several independent experiments. Results were quite similar to those for the specialists *P. rapae* and *P. brassicae*. Induced *BoLOX* transcript level as assessed by RT-PCR was apparent after 24 h of feeding by one *M. brassicae* caterpillar in the local leaf; after 72 h and 7 days, *BoLOX* transcripts were clearly present in both the local and systemic leaf (data not shown).

Tetranychus urticae spider mites. After an infestation with at least 50 spider mite females for 3 days, the damage to plants by spider mites was different compared with feeding damage inflicted by locusts and caterpillars. No clearly visible damage symptoms were observed up to 3 days after infestation (Fig. 4E). After 7 days, white spots were visible where the spider mites had emptied the content of parenchymous cells. On average, relative *BoLOX* transcript levels were increased 487- and 120-fold compared with control plants after 3 and 7 days, respectively (Fig. 8). *BoLOX* transcript levels were similar after 3 and 7 days of spider-mite infestation ($P > 0.05$).

Myzus persicae aphids. Green peach aphids (*Myzus persicae*) are generalists that feed on phloem sap. In our studies, *M. persicae* were allowed to feed for 7 days on the plants, resulting in a clear suppression of growth rate of plants when compared with healthy control plants (Fig. 4F and G). Severe effects of aphids on plant growth also occur in other plants (Mornhinweg et al. 2006). However, no *BoLOX* RNAs were detected by RT-PCR (data not shown).

Accumulation of *BoLOX* transcripts in response to mechanical damage.

After wounding plants by making three 2.5-cm-diameter punches in each of two fully expanded young leaves, there was no other obvious change in the remaining leaves of plants compared with control plants (Fig. 4H). *BoLOX* RNA was not detected 1, 6, and 24 h after mechanical damage by RT-PCR (data

not shown). *BoLOX* RNA could be detected 3 days after the infliction of mechanical damage in the local leaf, but not in the systemic leaf by RT-PCR (Fig. 9A). The relative *BoLOX* transcript level in the local leaf was significantly increased 141-fold.

Fig. 3. Quantitative real-time polymerase chain reaction of *Brassica oleracea* lipoxygenase (*BoLOX*) gene expression 24 h after exposure of *B. oleracea* plants to different jasmonic acid (JA) concentrations. JA-induced transcripts were quantified in three independent experiments at concentrations of 0, 10, 100, and 1,000 μM JA. *BoLOX* transcript levels are presented relative to transcript levels of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) after application of JA. Error bars indicate standard error of the mean ($n = 3$). Data points marked with different letters are significantly different (Kruskal-Wallis test, $\alpha = 0.05$).

Fig. 2. Phylogenetic tree of lipoxygenase (*LOX*) genes from different plant species. Accessions for LOX amino sequences represented in this tree are *Arabidopsis thaliana* AtLOX1(NM 104376); *A. thaliana* AtLOX2 (L23968); *A. thaliana* AtLOX3 (AJ249794); *A. thaliana* AtLOX6 (AJ748537), *Solanum tuberosum* potato H1(X96405), *S. tuberosum* potato H3(X96406), *Nicotiana attenuata* NaLOX3 (AY254349), *Lycopersicon esculentum* TomLOXC (U37839); *L. esculentum* TomLOXD(U37840), *Glycine max* SoyLOX3 (X13302), *B. napus* BnLOX1fl (AY162142), and BnLOX2 fl (AY162143).

The level of *BoLOX* transcript level in the systemic leaf was similar to that in the undamaged control plants (Fig. 9B).

Accumulation of *BoLOX* transcripts in response to the bacterial pathogen *Pseudomonas syringae*.

Pseudomonas syringae is a virulent bacterial leaf pathogen that causes extensive chlorosis and necrotic spots (Whalen et al. 1991). *P. syringae* pv. *tomato* elicits a hypersensitive response in Chinese cabbage (Ryang et al. 2002). In our experiments, infiltration of cabbage plants with *P. syringae* pv. *tomato* DC3000 was carried out. Chlorosis symptoms in cabbage leaves were apparent at 48 h post infiltration (hpi,) (Fig. 4I). Necrotic spots were visible after 72 hpi (Fig. 4J). Furthermore, necrotic spots spread into larger leaf areas after 96 hpi (Fig. 4K and L). To analyze *BoLOX* RNA levels in response to bacterial infiltration, RNA was isolated from cabbage leaves at different time points after treatment with *P. syringae* pv. *tomato* DC3000 and subjected to RT-PCR analysis (Fig. 10). Before the appearance of chlorosis and necrosis, *BoLOX* transcripts accumulated. A transient increase was observed, starting even at 1 hpi in the local leaf, but *BoLOX* RNA levels were highest at 1 and 6 hpi in the systemic leaf. *BoLOX* transcripts reached maximal levels in both local and systemic leaves after 24 hpi and declined after 48 hpi. However, *BoLOX* transcript levels went up again after 72 and 96 hpi, respectively. Because *BoLOX* RNA was not detected 1, 6, and 24 h after the infliction of mechanical damage by RT-PCR (Fig. 9A), we assume that mechanical damage by the act of infiltration itself will not lead to accumulation of *BoLOX* RNAs because it is a minor wounding compared with the mechanical damage we applied. However, this still needs to be verified by investigating the effect of infiltra-

Infestation with one locust for 72 hours

Fig. 5. Quantitative real-time polymerase chain reaction of *Brassica oleracea* lipoxygenase (*BoLOX*) gene expression. Relative *BoLOX* transcript levels in local, locust-damaged leaves (L) and systemic, undamaged leaves (S) after infestation of plants with one first-instar locust per plant for 72 h. Error bars indicate standard error of the mean ($n = 3$). Data points marked with different letters are significantly different (Kruskal-Wallis test, $\alpha = 0.05$).

Fig. 4. Cabbage plants were infested with different herbivores, wounded by mechanical damage, or inoculated with a bacterial leaf pathogen. **A**, Overview of morphologically uniform plants used in experiments. **B**, Plant infested with one first-instar *Schistocerca gregaria* for 72 h. **C**, Plant infested with 32 first-instar larvae of *Pieris rapae* for 24 h. **D**, Plant infested with two first-instar larvae of *P. brassicae* for 24 h. **E**, Plants infested with *Tetranychus urticae* spider mites for 3 days. **F**, Untreated control plant (left) and plant infested with *Myzus persicae* aphids for 7 days (right). **G**, Plant infested with *M. persicae* after 7 days. **H**, Plant at 3 days after mechanical damage with a total three punches in each of two leaves. **I**, Leaf symptoms after inoculation with *Pseudomonas syringae* pv. *tomato* DC3000 for 48 h. **J**, Leaf symptom after inoculation with *P. syringae* pv. *tomato* DC3000 for 72 h. **K**, Leaf symptoms after inoculation with *P. syringae* pv. *tomato* DC3000 for 96 h. **L**, Overview of leaf symptoms after inoculation with *P. syringae* pv. *tomato* DC3000 for 96 h.

tion on *BoLOX* transcript levels. The pattern of *BoLOX* RNA accumulation in response to *P. syringae* was recorded in all three independent experiments.

DISCUSSION

Isolation and characterization of *BoLOX*.

LOXs compose a gene family with two subgroups. Class 2 LOXs carry a putative chloroplast transit peptide and are 13-LOXs that oxygenate linolenic acid at the 13th carbon atom (Feussner and Wasternack 2002). A 13-LOX is required for the first step in the biosynthesis of JA from linolenic acid (Feussner and Wasternack 2002; Wasternack et al. 2006). The class 2 LOXs include *AtLOX2* in *Arabidopsis* (Bell et al. 1995), *TomLOXD* in tomato (Heitz et al. 1997), *LOX-H1* and *LOX-H3* in potato (Royo et al. 1996), *ZmLOX10* in maize (Nemchenko et al. 2006), and *NaLOX3* in *Nicotiana attenuata* (Halitschke and Baldwin 2003) that are wound and jasmonate inducible. Antisense LOX expression of *AtLOX2*

or *NaLOX3* reduced JA accumulation (Bell et al. 1995; Halitschke and Baldwin 2003) and direct or indirect defense (Halitschke and Baldwin 2003; Van Poecke and Dicke 2002), thus showing the involvement of these LOXs in the biosynthesis of JA. Here, we have sequenced a *LOX* from *B. oleracea* (*BoLOX*) that codes for a protein with a putative chloroplast transit peptide. Phylogenetic analysis shows that the sequence of this *LOX* is closely related to that of other LOXs from brassicaceous plants, among which is *AtLOX2* that codes for a protein involved in JA biosynthesis. Just like other class 2 LOXs, *BoLOX* RNA levels also are elevated after treatments by JA and wounding. Thus, *BoLOX* shares characteristics with many *LOX* genes in class 2 that code for enzymes involved in an early step in JA biosynthesis. JA mediates direct (van Dam et al. 2004) as well as indirect defenses against insects in *B. oleracea* (Bruinsma et al. 2007). Direct defense of *B. oleracea* includes the induction of secondary metabolites such as glucosinolates (van Dam et al. 2004) and indirect defense of *B. oleracea* includes the production of parasitoid-attracting vola-

Fig. 6. Effect of caterpillar density of feeding on *Brassica oleracea* lipoxigenase (*BoLOX*) transcripts. **A**, Effect of number of first-instar *Pieris brassicae* or *P. rapae* larvae that infested a leaf on transcript levels of *BoLOX* (upper bands) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (lower bands) genes as assessed by reverse-transcriptase polymerase chain reaction in local (L) and systemic (S) leaves. Numbers indicate the number of larvae that fed on the local leaf for 24 h. The housekeeping gene *GAPDH* was used to indicate equal loading. **B**, Relative transcript levels of *BoLOX* gene in local (L) and systemic (S) leaves after infestation of one first-instar *P. rapae* larva on the local leaf for 24 h. Error bars indicate standard error of the mean ($n = 3$). Data points marked with different letters are significantly different (Kruskal-Wallis test, $\alpha = 0.05$).

tiles (Mattiacci et al. 2001; Scascighini et al. 2005; Steinberg et al. 1992). Therefore, *BoLOX* RNA accumulation was correlated with the dynamics in the early steps in the induction of defenses in *B. oleracea* in other studies.

***BoLOX* transcript accumulation in response to herbivory and JA.**

We have extensively assessed the *BoLOX* transcript patterns with real-time quantitative PCR in response to different attackers and for different densities and different time points. These analyses show that *BoLOX* transcripts are upregulated from undetectable levels in undamaged plants to high levels after herbivory by each of three different caterpillar species (the specialists *Pieris rapae* and *P. brassicae* and the generalist *Mamestra brassicae*), a locust (*Schistocerca gregaria*), a spider mite (*Tetranychus urticae*), and JA. It seems that the maximum transcript levels assessed after caterpillar feeding (up to 10,000-fold more than in the control) are higher than the levels recorded in response to locust and spider-mite damage. Mechanical wounding also increased *BoLOX* transcript levels, but to lower levels than after herbivory. However, mechanical wounding was applied in a single bout whereas herbivory continued for a period ranging from 1 h to 7 days depending on species and experiment. It would be interesting to investigate *BoLOX* RNA levels in response to mechanical damage that carefully simulated herbivory, such as with the MecWorm robot (Mithofer et al. 2005).

BoLOX transcripts are induced locally as well as systemically. The systemic response is weaker than the local response when the plant is damaged by caterpillars or locusts whereas the systemic response is stronger than the local response when the plant is infested with the bacterial pathogen *Pseudomonas syringae*. For caterpillar-induced defenses, this agrees with the finding that induced indirect defense occurs both locally (Steinberg et al. 1992) and systemically (Mattiacci et al. 2001). It should be noted that the systemic leaf in our study is the leaf directly under the infested leaf, whereas the systemic leaf in the study by Mattiacci et al. (2001) is the leaf above the infested leaf.

Dynamics of *BoLOX* RNA accumulation patterns.

The transcript levels of *BoLOX* are highly correlated with the applied JA concentration. Although its accumulation is also dependent on caterpillar density and herbivory duration, the increase in *BoLOX* transcripts is restricted mainly to the lowest caterpillar densities and the shorter durations of herbivory. This corresponds well to the report that 3 to 72 h of feeding by five

Fig. 7. Effect of caterpillar feeding time on *Brassica oleracea* lipoxygenase (*BoLOX*) transcript levels. Relative *BoLOX* transcript levels in local (L) and systemic (S) leaves after infestation of a local leaf with one first-instar *Pieris brassicae* larva during different time periods. Error bars indicate standard error of the mean ($n = 3$). Data points marked with different letters are significantly different (Kruskal-Wallis test, $\alpha = 0.05$).

Fig. 8. Effect of spider mite infestation on *Brassica oleracea* lipoxygenase (*BoLOX*) gene expression. Relative *BoLOX* transcript levels after spider mite infestation for 3 or 7 days. Error bars indicate standard error of the mean ($n = 3$). Data points marked with different letters are significantly different (Kruskal-Wallis test, $\alpha = 0.05$).

first-instar *Pieris rapae* larvae induced a clear increase in JA levels in *Arabidopsis* (De Vos et al. 2005). Real-time quantitative PCR revealed that the fast increase in *BoLOX* transcript levels was induced by even a single *Pieris* caterpillar (i.e., already after 1 h of feeding). Although JA levels were not measured in

Fig. 9. Effect of mechanical wounding on *Brassica oleracea* lipoxygenase (*BoLOX*) gene expression in local (L) and systemic (S) leaves. **A**, *BoLOX* transcript levels in undamaged control plants (C), and in damaged local (L) and undamaged systemic (S) leaves of plants that had been mechanically damaged 3 days earlier. The housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase (*GAPDH*) was used to indicate equal loading. Data for three independent replicate plants. **B**, Relative *BoLOX* transcripts 3 days after mechanical damage. Error bars indicate standard error of the mean ($n = 3$). Data points marked with different letters are significantly different (Kruskal-Wallis test, $\alpha = 0.05$).

B. oleracea, by comparison with *Arabidopsis* and *N. attenuata*, it is presumed that this result may reflect the JA burst at the early stages of wounding (i.e., after 1 h in *Arabidopsis* [McConn et al. 1997] and 35 min in tobacco [Halitschke and Baldwin 2003]). The transcript level in both local and systemic leaves did not increase at caterpillar densities exceeding two first-instar larvae feeding for 24 h. A period of 24 h of caterpillar feeding was needed to reach maximal transcript levels in both local and systemic leaves. During longer feeding duration, the expression level remained at the same level. This is comparable with the induction of potato *LOX-H1* (Royo et al. 1996). In contrast, potato *LOX-H3* and tobacco *NaLOX3* reach a maximum transcript level after 30 and 60 min, respectively (Halitschke and Baldwin 2003; Royo et al. 1996).

It is remarkable that *BoLOX* transcript levels did not significantly increase when the caterpillar density increased from 2 to 32 per leaf. However, induced indirect defense of *B. oleracea* in response to *Pieris* caterpillars is highly density dependent. With increasing caterpillar density in the range of 1 to 90 first-instar *Pieris* caterpillars, the relative attraction of parasitoids increases as well (Geervliet et al. 1998a). Within 24 h after the onset of caterpillar feeding, the *BoLOX* transcript level is increased to a limit value. The induction of indirect defense in *B. oleracea* in response to caterpillar feeding (50 second-instar *P. brassicae* caterpillars) also is known to increase within hours to a maximum value (Scascighini et al. 2005). Parasitic wasps that attack the caterpillars are increasingly attracted several hours after the start of herbivory; the main increase in volatile emission during the early hours because herbivory occurs in green leaf volatiles such as (Z)-3-hexenol and (Z)-3-hexen-1-yl acetate (Scascighini et al. 2005) that are JA inducible (Dicke et al. 1999; Kessler and Baldwin 2001). The LOX concentration in the leaf may not be the limiting step after a certain transcript level has been reached. The amount of substrate, linolenic acid, is likely to determine the level of defense expression. Indeed, feeding of linolenic acid to plants induces defense expression (Koch et al. 1999).

BoLOX transcripts also were upregulated in response to infestation of the bacterial pathogen *Pseudomonas syringae*, but not in response to the aphid *Myzus persicae*. The aphids had been feeding for 7 days (i.e., much longer than, for instance, the caterpillars). Thus, the absence of *BoLOX* transcripts indicates that the aphids did not induce this gene, which is confirmed in other *B. oleracea* genotypes (C. Broekgaarden, M. Dicke, and B. Vosman, unpublished data). It is interesting to note that the caterpillar *Pieris rapae* and the bacterium *Pseudomonas syringae* induce the biosynthesis of JA in *Arabidopsis*, whereas the aphid *M. persicae* does not (De Vos et al. 2005). This correlates with their effect on the transcript level of JA-responsive *BoLOX*, as reported here. However, the fact that a certain attacker induces JA does not imply that all JA-

Fig. 10. Time course of *Brassica oleracea* lipoxygenase (*BoLOX*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene expression in pressure-infiltrated local leaves (L) and noninfiltrated systemic leaves (S) that were harvested at indicated time points after pathogen infiltration. Pressure infiltration was performed by infiltrating one fully expanded young leaf with a solution of 10 mM MgSO_4 (control) or with a bacterium suspension of an optical density at 600 nm = 0.1 of virulent pathogen *Pseudomonas syringae* pv. *tomato* DC3000. In total, three experiments were performed on separate dates with similar results. The housekeeping gene *GAPDH* was used to indicate equal loading.

responsive genes are upregulated (De Vos et al. 2005). Cross-talk with other signaling pathways may modulate the effect of JA (Bostock 2005; De Vos et al. 2006; Thaler et al. 2002). In *Arabidopsis*, *P. syringae* does not induce *AtLOX2* within 24 h when assessed with a full-genome microarray, despite the observation that *P. syringae* induces considerable increases in JA levels (De Vos et al. 2005). It will be interesting to investigate *AtLOX2* expression with the much more sensitive method of real-time quantitative PCR.

Induced defenses of *B. oleracea*.

B. oleracea can be attacked by a range of arthropod herbivores, including caterpillars, aphids, grasshoppers, and beetles (Root 1973). Induced defenses of *B. oleracea* have been reported in response to aboveground and belowground herbivory (van Dam et al. 2004). Here, we have shown that *BoLOX* is upregulated in response to different herbivorous arthropods. The only herbivore that did not induce *BoLOX* RNA accumulation was the aphid *M. persicae*. These data correspond to data on JA induction by these herbivores in other plant species. The caterpillar *Pieris rapae* induces JA in *Arabidopsis* (De Vos et al. 2005) and the spider mite *T. urticae* induces JA in tomato and Lima bean plants (Engelberth et al. 2001; Kant et al. 2004), whereas the aphid *M. persicae* did not induce JA in *Arabidopsis* (De Vos et al. 2005).

A range of herbivores are known to induce defenses in *Brassica* spp., such as the cabbage root fly (*Delia radicum*) (van Dam and Raaijmakers 2006); the caterpillars *Plutella xylostella* (Lu et al. 2004; Vuorinen et al. 2004), *Pieris rapae*, *P. napi*, *P. brassicae* (Geervliet et al. 1996), and *Mamestra brassicae* (Geervliet et al. 1996); the flea beetle *Phyllotreta cruciferae* (Palaniswamy and Lamb 1993); and the leaf beetle *Phaedon cochlearea* (Rostas and Hilker 2002b). Different caterpillar species induce similar, though not identical, indirect defense in *B. oleracea* (Geervliet et al. 1996, 1998b). Moreover, pathogens also induce defenses in *Brassica* spp. (e.g., the bacterium *Xanthomonas campestris* [Pastuglia et al. 1997] or the fungi *Alternaria brassicae* [Rostas and Hilker 2002a, 2003] and *Sclerotinia sclerotiorum* [Li et al. 1999]).

Crosstalk between signaling pathways is well known to influence phenotype expression and, thus, the interaction between pathogens and herbivores (Stout et al. 2006). The phytopathogen *Pseudomonas syringae* induces the phytohormones JA, salicylic acid, and ethylene in *Arabidopsis*, and a subset of JA-inducible genes are upregulated as a result (De Vos et al. 2005). In order to understand the plant-mediated interactions between phytopathogens and herbivorous insects, it is important to analyze the underlying mechanisms. For instance, investigating the mechanisms of *Pieris rapae*-induced resistance to phytopathogens unraveled new ways in which herbivorous insects influence plant-pathogen interactions, such as through priming of pathogen-inducible defense (De Vos et al. 2006).

Conclusion.

We have cloned a *B. oleracea* *LOX* gene that likely is involved in JA biosynthesis, and quantified its transcript levels after various treatments and time intervals. Our data show that *BoLOX* transcripts accumulate rapidly after various biotic stresses. The involvement of *BoLOX* in mediating interactions between *B. oleracea* and its attackers will be further investigated in molecular ecological studies on multitrophic interactions.

MATERIALS AND METHODS

Plants.

Brussels sprouts (*B. oleracea* var. *gemmifera* cv. Cyrus) were grown in a greenhouse (50 to 70% relative humidity [RH], 20 to

30°C, 16:8 h L/D [h of light and darkness, respectively]). Morphologically uniform plants with six to eight leaves were used in all experiments (Fig. 4A). Plants were placed in a climate chamber (70 ± 10% RH, 23 ± 1°C, 16:8 h L/D) for herbivore infestation or pathogen inoculation.

Herbivores.

Different herbivores were used in gene expression experiments. The caterpillars *P. rapae* (small cabbage white), *P. brassicae* (large cabbage white), and *Mamestra brassicae* (cabbage moth) were reared routinely on cabbage plants (*B. oleracea* var. *gemmifera* cv. Cyrus) at 21 ± 1°C, 50 to 70% RH, 16:8 h L/D). *Schistocerca gregaria* (desert locust) was reared routinely on a mixture of rye, cabbage leaves, and grass at 33 ± 2°C, 30 to 40% RH, 16:8 h L/D. These herbivore species are leaf-chewing insects. Mesophyll-feeding *T. urticae* (two-spotted spider mites) were reared on lima bean (*Phaseolus lunatus*) plants grown in a greenhouse (50 to 70% RH, 20 to 3°C, 16:8 h L/D). Phloem-feeding *Myzus persicae* (green peach aphids) were maintained on *B. chinensis* L. cv. Granaat in a greenhouse (50 to 70% RH, 20 to 30°C, 16:8 h L/D). The 16-h light period prevented sexual reproduction, keeping the aphid population clonal.

Bacteria.

The leaf pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (Pieterse et al. 1996) was cultured in liquid King's medium B (King et al. 1954) at 28°C. After we collected the cells by centrifugation, bacterial cells were resuspended in 10 mM MgSO₄ and adjusted to an optical density at 600 nm = 0.1 as described by Ryang and associates (2002).

RNA isolation.

Plants were subjected to different attackers for specified periods of time (discussed below). All harvested leaf material was directly frozen in liquid nitrogen, and stored at -80°C for further RNA extraction.

Total RNA isolation from control and treated *Brassica* leaves was performed according to the method described by Zheng and associates (2005) with minor modifications. Briefly, 0.75 ml of RNA extraction buffer (100 mM Tris pH8.5, 100 mM NaCl, 20 mM EDTA, and 1% Sarkosyl) with β-mercaptoethanol was added to each fine powder sample. An equal volume of phenol was added. After centrifugation, the aqueous phase was collected, and phenol/chloroform added followed by ethanol precipitation. The dried RNA pellet was dissolved by adding 50 µl of DNase/RNase-free diethylpyrocarbonate-treated water. The concentration of RNA was measured with a BIO-RAD SmartSpec 3000.

cDNA cloning of constitutive glyceraldehyde-3-phosphate-dehydrogenase and induced *BoLOX* gene.

First-strand cDNA was synthesized by following the manufacturer's protocol (Invitrogen, Leek, The Netherlands) and using 5 µg of RNA from fully expanded leaves of *B. oleracea* plants that had been infested with *Pieris rapae* larvae for 24 h in a total volume of 40 µl. Degenerate primers for *LOX* were designed from conserved regions of *Arabidopsis thaliana* *AtLOX1* (Melan et al. 1993) and *AtLOX2* (Bell and Mullet 1993), tomato *TomLOXC* and *TomLOXD* (Heitz et al. 1997), potato H1 (*LOX2*) and H3 (*LOX3*) (Royo et al. 1996), tobacco *LOX3* (Halitschke and Baldwin, 2003), and soybean *LOX3* (Yenofsky et al. 1988): the forward primer used was 5'-TDGCHGGHBTNAAYCC-3' and the reverse primer used was 5'-CTGTGYGGATCYTCMGTTGGC-3'. Because *GAPDH* was proven to be a good housekeeping gene in sugarcane (Iskandar et al. 2004), is active in all mammalian tissues, and

frequently is used as housekeeping gene in expression studies (Carraro et al. 2005), degenerate primers for *GAPDH* were designed from DNA sequence alignment of *GAPDH* sequences from several monocot and dicot species available in GenBank: forward primer 5'-AGRAACCCTGARGADATYCCRTGGG-3' and reverse primer 5'-CCAGCCTTGGCRTCRAARATGC T-3'. PCR was performed in a total of 50 µl containing 0.2 µM primers, 0.2 mM dNTPs, 0.4 units of Super Taq DNA polymerase (Invitrogen), 1× PCR buffer (Invitrogen), and 2 µl of first-strand cDNA. Temperature programs were: 2 min at 94°C; 40 cycles of 1 min at 94°C, 1 min at 45°C, and 1 min at 72°C; and 10 min at 72°C. The purification, ligation, and bacterial transformation of the PCR products was done according to the manufacturer's protocol. Ampicillin-resistant colonies were identified and plasmid DNA was isolated using a miniprep kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. Plasmids were selected which showed inserts of the expected size after digestion with *NcoI* and *Sall*. Expected inserts based on size were used for sequencing. Sequencing was performed in an ABI PRISM 310 automated DNA sequencer (Perkin Elmer, Foster City, CA, U.S.A.).

5'- and 3'-RACE of induced *LOX* gene.

Cloning of 5' and 3' ends of the inducible *LOX* gene was accomplished by RACE using the BD SMART RACE cDNA Amplification Kit (BD Bioscience Clontech, Palo Alto, CA, U.S.A.) following the manufacturer's protocol. 3'-RACE-Ready cDNA and 5'-RACE-Ready cDNA were synthesized from 1 µg pf RNA from *P. rapae*-infested leaves. *BoLOX* gene-specific primers were designed based upon the above-mentioned partial sequence. The criteria for these gene specific primers were 23 to 28 nucleotide, 50 to 70% GC, and temperature >70°C, enabling the use of touchdown PCR. The forward gene-specific primer is GSF1: 5'-GGAGTGGTCCACAGTCA AGGGCACTG-3' and reverse gene-specific primer is GSR1: 5'-CCCCCTGCTGATGAGGTCTGCAGGTA-3'. The gene-specific primers designed here produced overlapping 1,030-bp products. For 5'-RACE or 3'-RACE, a total volume of 50 µl was used in the PCR reaction and touchdown PCR was performed for 2 min at 94°C; 5 cycles of 30 s at 94°C and 3 min at 72°C; 5 cycles of 30 s at 94°C, 30 s at 70°C, and 3 min at 72°C; and 25 cycles of 30 s at 94°C, 30 s at 68°C, and 3 min at 72°C based on the recommended protocol. The 5'- and 3'-RACE products were purified, ligated into pGEM-T vector (Promega Corp., Madison, WI, U.S.A.), and sequenced as explained above.

Sequence analysis.

For sequence analysis we used the Vector NTI software (Advance 9.1; Invitrogen, life science software). For contig building, the CAP3 Sequence Assembly Program was used (Huang and Madan 1999). Sequence relatedness was analyzed using ClustalX 1.83 using the neighbor-joining method, and visualized using TreeView. Subcellular localization prediction for protein was performed with the iPSORT prediction program.

Reverse transcription followed by quantitative real-time PCR (real-time RT-PCR) for gene expression.

All RNA samples (10 µg of total RNA) were treated with 1 µl of RQ1 RNase-free DNase (Promega Corp.) with 1× DNase buffer in a total volume of 50 µl at 37°C for 10 min to remove genomic DNA. The samples were purified by extraction with equal volumes of phenol/chloroform and chloroform followed by ethanol precipitation as described for the RNA isolation. After purification, the RNA concentration was measured again using a NanoDropND-100 spectrophotometer. The iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, U.S.A.) was

used for synthesis of cDNA from 1 µg of total RNA in a total volume of 20 µl. The reaction was incubated at 25°C for 5 min and 42°C for 30 min. The reaction was stopped by incubating at 85°C for 5 min. Then, 80 µl of water was added to each sample to make the final 100 µl of cDNA solution. The gene-specific primers of *BoLOX* and *GAPDH* were designed with the Beacon Designer software (Premier Biosoft International, Palo Alto, CA, U.S.A.) set to an annealing temperature of 60°C. *BoLOX* primers were F-*BoLOX* (5'-ACTTTCCCGTCC CGTTCTTGG-3') and R-*BoLOX* (5'-GATTGTCGTGCCCCGT GAATGC-3'). Its predicted length was 175 bp. *GAPDH* primers were F-*GAPDH* (5'-GCTACGCAGAAGACAGTTGATGG-3') and R-*GAPDH* (5'-TGGGCACACGGAAGGACATAC-3'). Its predicted length was 163 bp. These primer sequences were blasted against the National Center for Biotechnology Information nucleotide and expressed sequence tag database to ascertain that they are not homologous to other genes. Several primer concentrations ranging from 200 to 800 nM were used to determine the optimal primer concentrations; 400 nM for both F-*BoLOX* and R-*BoLOX* and 800 nM for both F-*GAPDH* and R-*GAPDH* were optimal. For PCR amplification efficiency, gradient annealing temperatures from 56 to 66°C were checked in advance. This resulted in the selection of 56°C as the optimal annealing temperature for both PCRs. One sample was chosen for making a five-log dilution series, on the basis of highest expected *BoLOX* transcript level. In each 96-well plate, this dilution series was included. The RT-PCR analyses of reference and target gene transcripts in cDNA samples were conducted in the iCycler IQ real-time PCR detection system (Bio-Rad). A 25-µl PCR reaction was prepared containing 1 µl of template cDNA, 1 µl each of forward and reverse primer, 9.5 µl of water, and 12.5 µl of 2× iQ SYBR green Supermix (Bio-Rad). The final amount of cDNA template assayed was equivalent to 10 ng of RNA. All samples were amplified in duplicate assays under the following conditions: 95°C for 3 min for 1 cycle, followed by 40 cycles of 95°C for 15 s and 45 s at 56°C, with data collection at 56°C. The PCR products for each primer set also were subjected to melt-curve analysis. The melt-curve analysis ensured that the resulting fluorescence originated from a single PCR product and did not represent primer dimers formed during the PCR or nonspecific product. No-template control as water and minus RT (10 ng of RNA) also were included to detect any spurious signals arising from amplification of any DNA contamination or primer dimer formed during the reaction. Direct detection of the PCR product was measured by monitoring the increase in fluorescence caused by the binding of the SYBR green dye to double-stranded DNA. The *BoLOX* expression relative to *GAPDH* expression was quantified by comparing the threshold cycle for each PCR to their respective dilution series and dividing the resulting quantities. The *BoLOX/GAPDH* ratios for all samples were related to the ratio for untreated plants, which was set to 1. The mean efficiencies and standard errors of the means of the PCRs were $94.8 \pm 1.6\%$ for *BoLOX* and $97.6 \pm 2.3\%$ for *GAPDH*, based on the dilution series in eight PCR runs.

Quantitative RT-PCR data were analyzed with the Kruskal-Wallis test ($\alpha = 0.05$).

Analysis of *BoLOX* transcript patterns.

For a few experiments, RT-PCR was carried out. First-strand cDNA was synthesized as mentioned above. The PCR program used for gene expression was 2 min at 94°C; 5 cycles of 30 s at 94°C and 3 min at 72°C; 5 cycles of 30 s at 94°C, 30 s at 70°C, and 3 min at 72°C; and 15 cycles of 30 s at 94°C, 30 s at 68°C, and 3 min at 72°C. The gene-specific primers GSF1 and GSR1 produced 1,030-bp products. Touchdown PCR also was used for the housekeeping gene *GAPDH* as loading control.

The PCR program was 2 min at 94°C; 5 cycles of 30 s at 94°C and 3 min at 72°C; 5 cycles of 30 s at 94°C, 30 s at 70°C, and 3 min at 72°C; and 10 cycles of 30 s at 94°C, 30 s at 68°C, and 3 min at 72°C. *GAPDH*-specific primers (forward primer: 5'-CACTGACAAGGACAAGCTGCTGCT-3'; reverse primer: 5'-CGGCTCTTCCACCTCTCCAGTCCTTC-3') produced 299-bp products. For the following gene-expression experiments, local and systemic leaf definition, number of replications per treatment, sampling, RNA isolation, first-strand cDNA synthesis, and PCR conditions were the same as mentioned above unless otherwise specified.

Different densities of *P. rapae* and *P. brassicae* caterpillars.

To investigate *BoLOX* transcript levels in cabbage plants that had been subjected to different caterpillar numbers, 1, 2, 4, 8, 16, or 32 first-instar larvae of *P. rapae* or *P. brassicae* were used to infest one of the youngest fully expanded leaves for 24 h. Control plants were placed in the same climate chamber without contact with caterpillars. Care was taken to avoid wounding of plants during transfer of larvae to the plants. There were at least three replicate plants for each dose treatment. The infested and damaged youngest, fully expanded leaf was denoted as local leaf, while one undamaged leaf directly below the infested leaf was denoted as systemic leaf. Before sampling, we checked that the larvae were still feeding on the leaf on which they had been released. In most cases, caterpillars had stayed on the initial leaf; otherwise, the plant was discarded. Subsequently, the larvae were removed from the local leaf and the leaf was cleaned with wet tissue.

Different periods of feeding

by *P. rapae*, *P. brassicae*, or *M. brassicae* caterpillars.

Cabbage plants were infested with a single first-instar larva of either *P. rapae* or *P. brassicae* on one of youngest fully expanded leaves for 1, 6, 24, 48, 72, or 96 h. Control plants were placed in the same climate chamber without contact with caterpillars. The same was done for a single caterpillar of *M. brassicae* and, here, infestation lasted 72 h or 7 days.

Infestation with *S. gregaria*.

One first-instar *S. gregaria* locust was transferred onto a cabbage plant placed in a cage. One plant was placed in each cage. A control plant also was placed into a cage in the same climate chamber without contact with *S. gregaria*. One of the youngest fully expanded leaves that received locust feeding was denoted as local leaf. The leaf below this local leaf had been covered with a plastic bag to prevent locust feeding and this undamaged leaf was denoted as systemic leaf. The infestation lasted for 72 h.

Infestation with *T. urticae*.

Adult female two-spotted spider mites were transferred to cabbage plants. Lima bean leaves with more than 50 adult female of spider mites were placed on a leaf of each cabbage plant. After the spider mites had moved onto the cabbage plant for 3 to 4 h, the lima bean leaves were removed. One of the youngest fully expanded leaves that had received visible spider mite damage was sampled 72 or 168 h after introduction of the mites. The mites were removed before sampling. Control plants were placed in the same climate chamber without contact with spider mites. Local and systemic leaves were not separated because spider mites distributed over the whole plant.

Infestation by *Myzus persicae*.

Cabbage plants were infested with *Myzus persicae* aphids by carefully transferring 40 nymphs and apterous adults to each plant with a fine brush. Infested and control plants were

placed in a climate chamber for 7 days. Because *M. persicae* reproduce asexually and produce larvae very quickly, they attained high densities on the whole plant, especially on young leaves, causing water stress, wilting, and reduced growth rate of the plant after 7 days of infestation (Fig. 4F and G). A fully expanded young leaf was sampled after the aphids had been feeding for 7 days and aphids were removed before sampling.

Mechanical wounding and treatment of plants with the phytohormone JA.

A cabbage plant was mechanically wounded by making 2.5-cm-diameter punches in the fully expanded young leaves using a cork borer. Two leaves were damaged and three punches were made in each of these leaves. The six punches were collected from each plant and the material was immediately frozen in liquid nitrogen and treated as control for this plant. At 1, 6, 24, and 72 h after wounding the leaves, damaged leaves and one leaf just below the damaged leaves were sampled as local and systemic leaves, respectively.

Intact plants were sprayed with 20 ml of an aqueous solution of the phytohormone JA (Sigma, St. Louis) at concentrations of 10, 100, and 1,000 μ M JA. The solutions contained 0.1% Tween 20. Control plants were sprayed with 20 ml of water containing 0.1% Tween 20. All plants were maintained in a climate chamber for 24 h.

Inoculation of bacterial pathogen *Pseudomonas syringae*.

One fully expanded young cabbage leaf per plant was inoculated with the bacterial suspension by syringe infiltration as described by (Ryang et al. 2002). This pressure-infiltrated leaf was denoted as local leaf and one untreated leaf just below this local leaf was designated as systemic leaf. Mock inoculations were performed with 10 mM MgSO_4 in control plants. The local leaf was covered with a plastic bag to maintain 100% RH. Plants were incubated in a climate chamber (70% RH, 23°C, 16:8 h L/D). After 1, 6, 24, 48, 72, and 96 h, the leaf samples were harvested.

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AUTHOR-RECOMMENDED INTERNET REOURCES

CAP3 Sequence Assembly Program website: pbil.univ-lyon1.fr/cap3.php
 University of Glasgow, Institute of Biomedical and Life Sciences Treeview webpage: taxonomy.zoology.gla.ac.uk/rod/treeview.html
 PSORT WWW server: psort.nibb.ac.jp