

Cloning and Identification of Expressed Plant Defense Genes Following RT-PCR Using the MasterAmp™ High-Fidelity RT-PCR Kit and Degenerate Primers

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Pathogen infection can activate several classes of pathogenesis-related (PR) genes in a wide range of plant species. However, very little is known about pathogen-induced defense responses in sugarbeet plants. In a collaborative effort with Barry Jacobsen's group (Montana State University) we are seeking to characterize the molecular and biochemical basis for induced defense responses against pathogens in sugarbeet leaves. Since very few genes have been isolated from sugarbeet, a necessary first step toward this end entailed isolating and cloning, by RT-PCR, a battery of PR gene transcripts from sugarbeet leaves to use as molecular probes to monitor defense response activation. Chitinase, peroxidase, and β -glucanase genes were initially selected as candidate markers because they have been previously demonstrated as reliable markers for induced defense responses in other plant species. The MasterAmp™ High Fidelity RT-PCR Kit was chosen because of its excellent sensitivity, and its utilization of a blend of high fidelity PCR enzymes to ensure accurate sequence of the RT-PCR products.

Sugarbeet leaves were treated with either the plant defense response elicitor benzothiadiazole (BTH) or water (negative control). Total RNA was isolated by a modified phenol-SDS procedure followed by LiCl precipitation. This RNA was used as template for RT-PCR.

RT-PCR primer design

The degenerate primer (poly-T lok) consisting of 20 T residues ending in either A, C, or G at its 3'-end was used as the reverse transcription (RT) primer. Since very little sequence information is available for sugarbeet genes, PCR primers for each RNA transcript were designed by first comparing amino acid sequences of comparable known genes from diverse plant species. We identified conserved domains for each of the three model response markers and then made internal degenerate oligonucleotide primers for the subsequent PCR step. The degree of degeneracy for the internal primers ranged from 128X-512X.

Figure 1. Comparison of the sugarbeet β -1, 3 glucanase RT-PCR product produced using the MasterAmp™ High Fidelity RT-PCR Kit and a competitor's kit. RT-PCR was performed using highly degenerate primers and total RNA purified from benothiadiazole-treated sugarbeet leaves.

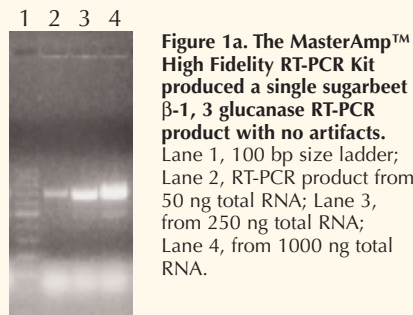
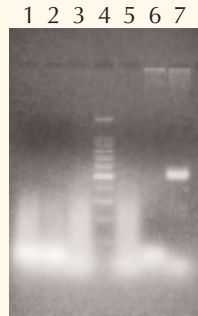


Figure 1a. The MasterAmp™ High Fidelity RT-PCR Kit produced a single sugarbeet β -1, 3 glucanase RT-PCR product with no artifacts. Lane 1, 100 bp size ladder; Lane 2, RT-PCR product from 50 ng total RNA; Lane 3, from 250 ng total RNA; Lane 4, from 1000 ng total RNA.

Figure 1b. A competitor's RT-PCR Kit produced no discernable sugarbeet β -1, 3 glucanase RT-PCR products. Lane 1, RT-PCR product from 25 ng total RNA; Lane 2, from 250 ng total RNA; Lane 3, from 1000 ng total RNA; Lane 4, 100 bp ladder; Lane 5, negative control (no added RNA); Lane 6, negative control (no added primers); Lane 7, positive control (manufacturer's kit control).



RT-PCR Method

RT-PCR was performed according to the standard one-step, one-tube continuous RT-PCR protocol described in the MasterAmp High Fidelity RT-PCR Kit product literature. First, 300 ng of total RNA from BTH-treated plants, 150 pmoles (3 μ M final concentration) of poly-T lok primer, and an added RNase inhibitor were combined and heated to 65° C for 5 minutes followed by immediate immersion in an ice-water bath. After 3-5 minutes on ice, the remainder of the RT-PCR reagents (MasterAmp 2X RT-PCR PreMix, MMLV-RT Plus, MasterAmp TAQurate™ DNA Polymerase Mix and the transcript-specific PCR Primer 2) were added. Optimum first strand synthesis (reverse transcription) for our model plant system was performed at 45° C for 40-45 minutes. The reaction was then transferred to a 95° C heat block for 2 minutes followed by

36-38 PCR amplification cycles using cycling conditions optimized for each primer pair. The final RT-PCR products were cloned and the DNA sequences of each determined.

Results

Figure 1a shows a typical RT-PCR result obtained using EPICENTRE's MasterAmp High Fidelity RT-PCR Kit. Even when performing RT-PCR with degenerate primers, the MasterAmp High Fidelity Kit produced complete RT-PCR product without artifacts. In contrast, using the same primers and template, an RT-PCR kit from another supplier did not produce any identifiable RT-PCR product except in the control reaction (Figure 1b). The identities of the cloned RT-PCR products produced using the MasterPure High Fidelity RT-PCR Kit were confirmed by DNA sequencing. Sequence comparison of the cloned sugarbeet genes to related homologues from other plant species revealed a surprising degree of divergence in the sugarbeet sequences.

We used the cloned sequences as probes in Northern analysis and confirmed that the three marker genes were induced systemically after treatment of sugarbeet leaves with BTH (data not shown). In contrast, no induction of the marker genes was seen in leaves treated with water. Complete results of this work will be published elsewhere.

MasterAmp™ High Fidelity RT-PCR Kit

RF91025	25 Reactions
RF910100	100 Reactions

Contents:

MMLV-RT Plus, MasterAmp™ TAQurate™ DNA Polymerase Mix, MasterAmp™ 2X RT-PCR PreMix (includes dNTPs), MasterAmp™ 10X PCR Enhancer, Random Nonamer Primer, Oligo (dT)₁₈ Primer, Control Template and Primer Mix, Sterile Water