Nucleotide Sequence Analyses of a Sugarbeet Genomic NPR1-Class Disease Resistance Gene

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ABSTRACT

Disease resistance in *Arabidopsis thaliana* is centrally controlled by the *NPR1* gene, which modulates multiple disease response pathways. A homolog of *NPR1* was isolated from *Beta vulgaris* as a first step in deducing the potentially similarly important role of this gene for sugarbeet disease resistance. Most structural and nucleotide sequence features of Arabidopsis *NPR1* were similar with the isolated beet *NPR1* homolog, including utilization of an unusual transcription start site motif, similarly positioned BTB/POZ and ankyrin repeat domains, and the coding sequence interrupted by three similarly positioned introns. In contrast, the length of the sugarbeet *NPR1* homolog was three-fold greater than that of Arabidopsis and most of the size difference between beet and Arabidopsis occurred in non-coding DNA sequences such as introns. The coding sequence of the sugarbeet *NPR1* homolog has 100% nucleotide sequence identity with the full-length *Beta vulgaris NPR1* cDNA sequence AY640381.

Additional Key Words: BAC library, gene discovery, gene structure, nucleotide sequence

INTRODUCTION

For beet (*Beta vulgaris* L.), model species have been instrumental in identifying molecular markers that co-segregate with agronomic traits (Schneider et al., 1999; 2002), predicting gene functions from nucleotide sequence (Herwig et al., 2002), recovering gene sequences for physiologically characterized beet proteins (Haagenson et al., 2006), and comparing gene synteny with unrelated species (Dominguez et al., 2003). A whole genome sequence for sugarbeet will allow comparative genomic approaches to agronomic gene discovery not currently available for beet. Full genome sequences are increasingly useful in predicting gene functions and shared physiological and agronomic processes involved in plant productivity.

Comparisons of beet genes with those from model plants, such as *Arabidopsis thaliana* (L.) Heynh., are useful if gene structure and function is generally conserved, an assumption that is generally considered valid for provisional assignments. Comparative structural analyses of genomic clones allow access to the predicted amino acid sequences of conserved proteins, but more importantly to the regulatory elements that control gene expression. For example, promoters conferring taproot expression patterns were recently confirmed by Oltmanns et al. (2006). Sequences in other genomic contexts (e.g. introns, terminators) have not been reported for native beet genes, with the exception of genomic sequences for a *Beta procumbens* C. Sm. translocation associated with nematode resistance in sugarbeet (Oberschmidt et al., 2003; Schulte et al., 2006).

Disease resistance in Arabidopsis is centrally controlled by the *NPR1* gene (**N**onexpressor of **P**athogenesis **R**elated proteins), which simultaneously modulates multiple responses to natural stimuli including the salicylic acid (as a positive effector)

and jasmonic acid (as a negative effector) defense response systems (Dong, 2004; Pieterse and van Loon, 2004). The *NPR1* gene was identified in a screen for mutants altered in response to pathogenesis and subsequently cloned and characterized (Cao et al., 1994; Delaney et al., 1995). Dynamic intracellular regulation of Arabidopsis NPR1 protein activity is controlled by changes in redox states, partly a result of the oxidative burst that converts inactive multimeric NPR1 proteins to active monomeric forms. Active NPR1 monomers accumulate in the nucleus, bind TGA-type transcription factors and ultimately activate the expression of Pathogenesis Related (PR) protein genes. Additional proteins interact with NPR1 in complexes that are mediated by specific protein-protein interaction domains within the NPR1 protein sequence (Ekengren et al., 2003; Despres et al., 2003; Mou et al., 2003; Thurow et al., 2005; Weigel et al., 2001; 2005; Xu et al., 2006).

Several examples of improved disease resistance resulting from over-expression of the *NPR1* gene are available (Cao et al., 1998; Chern et al., 2005; Fitzgerald et al., 2001). In Arabidopsis, transformants with higher levels of expression of *NPR1* had greater disease resistance against oomycete and bacterial pathogens. Fitzgerald et al. (2004) reported that over-expression of the Arabidopsis *NPR1* gene in rice led to a disease lesion mimic phenotype. Levels of disease resistance in tomato were enhanced using the *NPR1* gene from Arabidopsis (Lin et al., 2004) and in rice using a *NPR1* homologous gene from rice (Chern et al., 2005).

Manipulation of *NPR1* genes in crop plants has the potential to enhance plant resistance to microbial pathogens. Identification, isolation and annotation of a sugarbeet genomic segment carrying a gene homologous to *NPR1* is an important first step toward

improving disease resistance via manipulation of *NPR1* and its associated protein complexes in beets. In this paper, we report the full genomic nucleotide sequence of a *Beta vulgaris NPR1* homolog that shares many structural features with the full genomic sequence of the Arabidopsis *NPR1* gene and is identical to the beet NPR1 polypeptide sequence described by Bargabus-Larson and Jacobsen (2007) and is also similar to other plant NPR1 predicted coding sequences.

MATERIALS AND METHODS

A BAC library of sugarbeet hybrid US H20 (McGrath et al., 2004) was screened with primers designed on the sugarbeet cDNA sequence AY640381 deposited in GenBank (Lawton et al., 2004). AY640381, designated *NIM1* (synonymous with *NPR1*), predicts a protein homologous to the Arabidopsis *NPR1* GenBank accessions NM_105102 and AY088183. Several sets of potential gene-specific primers were evaluated for selective amplification of a particular segment(s) of AY640381. A 355-bp amplicon was produced using US H20 genomic DNA with primers 5'-ATG CTG TGG CAC ATT GTG AT-3' (forward) and 5'-CCT GCC TTT GCA AGA GAA AC-3' (reverse) on a PTC-100 Thermal Cycler (MJ Research, Inc., Waltham, MA) using FailSafe PCR (Epicentre Biotechnologies, Madison, WI) and standard PCR conditions (initial denaturation 94° C for 7 min, denaturation for 1 min at 94° C, annealing at 60° C for 3 min, primer extension for 3 min at 72° C, final cycle of 72° C for 7 min, followed at the end by a 4° C hold). PCR products were analyzed by electrophoretic staining of a 1.0% agarose gel visualized after ethidium bromide staining.

The US H20 sugarbeet BAC library (designated 'SBA', Amplicon Express, Pullman WA) was matrix pooled (Stormo et al., 2004). This allowed the identification of a

specific clone in two rounds of PCR. Initially a signal was identified within one of eight 4,608 BAC clone super-pools. Subsequently the specific clone was identified from among 36 matrix pools, each with 1,152 BAC clones, constructed from the super-pools. Ultimately a single BAC clone, SBA091H24, was identified and all further operations used this clone as the starting material. Clone confirmation employed the Genomiphi DNA Amplification Kit (product number 25-660-01, GE Healthcare, Piscataway, NJ) as per manufacturer directions, based on the methods of Dean et al. (2001). Briefly, ca. 1 ul of cells containing the BAC clone of interest was scraped from the frozen stock with a sterile pipet tip, added to 9 ul of sample buffer containing random hexamer primers, and heated to 95 °C for 3 min. To this was added 10 ul of Phi29 DNA polymerase and dNTP mix (proprietary concentrations, based on Dean et al., 2001), incubated isothermally at 30 °C for 20 hr, followed by heat inactivation of Phi29 at 65° C for 10 min, and 1 ul of this sample was used for traditional PCR.

BAC plasmid DNA was purified with the Qiagen Large Construct Kit (Qiagen Inc., Valencia, CA). BAC clone SBA091H24 was subcloned and sequencing of these subclones identified exons of the *Beta vulgaris NPR1* homolog, flanked by apparent intron sequences. The entire BAC clone was sequenced by the Genome Sequencing Center at The Washington University of St. Louis (St. Louis, MO). Shotgun cloning and sequencing of random subclones produced about 9.4x sequence coverage of SBA091H24 and complete sequence data was assembled using the phred/phrap suite. Subsequent analysis of the sequence data was performed using BLAST (Altschul et al., 1990), GenScan (Burge and Karlin, 1997) and the Lasergene v. 6 suite (DNASTAR, Madison, WI).

RESULTS AND DISCUSSION

Identification of an *NPR1*-like genomic clone

A BAC clone, SBA091H24, carrying an *NPR1* homologous gene was identified using primers designed based on *Beta vulgaris* cDNA accession AY640381 (Lawton et al., 2004). The predicted protein product of this sugarbeet cDNA had 52.4% amino acid identity and 69.3% amino acid similarity with the NPR1 protein of *Arabidopsis thaliana*. Arabidopsis has six *NPR*-like genes, and only *NPR1* and *NPR2* share significant amino acid identity (61.3%) compared with 36% identity with the next most similar paralog *NPR4*, also implicated in disease resistance (Liu et al., 2005). [Orthologous genes in gene families are often more conserved between species than are their corresponding paralogs within a given species.] Thus, the sugarbeet cDNA sequence predicts a protein product with similarity to the functionally defined Arabidopsis gene *NPR1*, as well as 100% polypeptide sequence identity to the experimentally defined sugarbeet *NPR1* amino acid residues from amino acid 82 to 578 of the predicted AY640381 coding sequence (Bargabus-Larson and Jacobsen, 2007). We have provisionally designated this sugarbeet gene as *BvNPR1-H1* (*Beta vulgaris NPR1-Homolog 1*).

The public nucleotide sequence database GenBank holds numerous ESTs (Expressed Sequence Tags derived from single pass sequencing of cloned messenger RNA) for *NPR1*-like genes from many plant species, however few are full length. Comparison of full-length *NPR1*-like genes from tomato (*Solanum lycopersicum* L.; 65.0% amino acid identity), tobacco (*Nicotiana tabacum* L.; 63.8% identity), Arabidopsis and *Brassica juncea* (L.) Czern. (47.9% identity) with the sugarbeet coding region shows striking overall similarity among these widely divergent species (Figure 1). For instance, amino

acid identities are frequent at numerous residues including the 129 amino acid BTB/POZ domain (47/129 = 36.4%; BLAST Score = 7.0 x 10^{-50}), and the 102 amino acid ankyrin repeat (56/102 = 54.9%, BLAST Score = 7.0 x 10^{-16}), as referenced against the sugarbeet *NPR1*. The BTB/POZ domain mediates protein dimerization and is present near the N terminus of some zinc finger proteins that act as transcriptional repressors (Zollman et al., 1994; Deweindt et al., 1995). Ankyrin repeats are tandemly repeated modules of about 33 amino acids that occur in numerous functionally diverse eukaryotic proteins and were experimentally proven to be involved in protein:protein interactions (Mosavi et al., 2004). Two other regions of contiguous identities are evident (residues 449-553 and 520-528, Figure 1), however these regions are not yet annotated for putative function(s). The predicted sugarbeet protein is comprised of 604 amino acids in total, slightly longer than the protein of Arabidopsis (593 residues).

Genomic sequence analysis and annotation

The genomic *BvNPR1-H1* gene described here encodes a predicted protein product 100% identical to that predicted from the *Beta vulgaris NPR1*-like (*NIM1*) cDNA sequence accession AY640381, which was used to devise the gene-specific primers. Interestingly, these primers also amplified identically-sized, single fragment amplicons of equally high intensity from each of the tested Caryophyllales species: beet, spinach (*Spinacia oleracea* L.), quinoa (*Chenopodium quinoa* Willd.), and ice plant (*Mesembryanthemum crystallinum* L.), suggesting strong conservation of these primer sites (data not shown). Phenetic comparisons show that *NPR1* coding sequences from phylogenetically related taxa cluster together (e.g. Solanacea, Brassicacea), and sugarbeet

occupies an intermediate position in this comparison of more distantly related core eudicot species (Figure 2).

BAC clone SBA091H24 was subcloned and sequenced to a depth of coverage of 9.4X, yielding ca. 150 kb of unique sequence in 22 contiguous regions. These 22 contigs have not yet been ordered. The largest contig (Contig 22) contained 38.5-kb of sequence putatively encoding two hypothetical genes, an integrase, and a heat shock protein as well as *BvNPR1-H1* (data not shown). No other predicted gene was found within 5 kb of *BvNPR1-H1*. The complete Contig 22 nucleotide sequence has been deposited in GenBank as accession DQ851167.

BvNPR1-H1 has four exons with 100% nucleotide identity as well as 100% amino acid identity in predicted protein product to the cDNA (GenBank accession AY640381). A search for additional transcripts among the *Beta vulgaris* Expressed Sequence Tag collection represented by the TIGR Sugar Beet Gene Index (McGrath unpublished, see Pertea et al., 2003) revealed three other transcript sequences with similarity to *BvNPR1-H1* in Tentative Consensus group TC1558. Unfortunately, none of these appears to represent an *NPR1* gene, as two contain an apparent sugarbeet unique polypeptide motif in Exon 1 (nucleotides 217-258; SDSFADAKIVVS; BQ588746 and BQ588928) and the other (BQ595462) has an Exon 4 motif present in Arabidopsis *NPR1* (nt 1-36; VELGKRFFPRCS), but none of these share other apparent similarity to *BvNPR1-H1*. Further, an apparent Exon 2 *NPR1*-unique motif embedded within the ankyrin motif (nt 445-477; TVLHVAAMRKEP) with identity between beet and Arabidopsis failed to recover any beet EST sequences in similarity searches. TC1558 did not align with *BvNPR1-H1*. Thus, functional information about *BvNPR1-H1* has yet to be deduced.

In silico analyses of *BvNPR1-H1* were used to compare and predict various shared and unique features of the sugarbeet genomic nucleotide sequence, comparing against the only other fully sequenced genomic *NPR1* dicot gene, that from Arabidopsis (GenBank accession AC066689 for *Arabidopsis thaliana* Chromosome 1, BAC F15H21.6, complete genomic sequence). All *BvNPR1-H1* exons showed similar polypeptide sequence similarities with Arabidopsis *NPR1* (Exon 1 = 49.2%, Exon 2 = 55.4%, Exon 3 = 45.7%, Exon 4 = 56.4%). Both *BvNPR1-H1* and Arabidopsis *NPR1* coding sequences, which are transcribed then processed to form mature mRNA for translation into protein, are interrupted by three introns (Figure 3). Intron 1 of *BvNPR1-H1* (1,957 bp) is 24.8 times longer than Intron 1 of Arabidopsis. Similarly, Introns 2 (1,430 bp) and 3 (780 bp) are 13.2 and 7.1 times longer than their Arabidopsis introns, respectively. No recognizable sequence motifs were found within Introns 1 and 2 of *BvNPR1-H1*. However, the 780 bp beet Intron 3 was similar to six beet ESTs in GenBank (*e* values $2x10^{-7}$ to $2x10^{-50}$, best alignment with GenBank accession BI643321) and 60 beet Genome Survey Sequences (*e* values $1x10^{-10}$ to $1x10^{-161}$, best alignment with GenBank accession ED026716), primarily from Bacterial Artificial Chromosome end sequences. A central 408 bp region of this predicted coding sequence (nt 5004 to 5410 from the putative start codon) appears to have the highest similarity among these GenBank accessions, with some variability observed. The function and significance, if any, of this moderately repetitive element apparently specific to *Beta vulgaris* is unknown.

Intron splice junctions were examined for *BvNPR1-H1* with respect to Arabidopsis *NPR1* represented by F15H21.6 (GenBank AC066689). Splice junctions in both species for Intron 1 followed the canonical Exon 1-GTnn/nnAG-Exon 2 boundaries. Alternative

splice junctions for Introns 2 and 3 were seen, with a 5' Exon-GGTA and 3' AT-rich region (>65% AT in *BvNPR1-H1*) within the 66 bp upstream of the splice junction (Ast, 2004; Lou et al., 1993). Curiously, in both species, the reading frame of Exon 2 is out of register (e.g. frame-shifted) by one nucleotide relative to the other exons, however the significance of this observation is moot when considering the protein structure.

Transcriptional promoter and terminator sites were found immediately upstream and downstream, respectively, of the predicted coding sequences of both beet and Arabidopsis *NPR1*. The canonical poly-adenylation site (AATAA) that signals the addition of a poly-A tail characteristic of eukaryotic messenger RNA was located 304 bp downstream of the stop codon in beet in the 3' untranslated region (UTR), with two observed in Arabidopsis at 209 and 254 bp downstream of its stop codon. The promoter regions were more interesting, with beet and Arabidopsis sharing many common features, in an inverted orientation (Figure 4). The W-box motif (e.g. TTGAC) is commonly found among genes involved in response to pathogens (Eulgem et al., 1999; Yu et al., 2001), and can function in an inverted orientation (Turck et al., 2004). However, in beet, the W-box is located on the non-transcribed strand, and a typical non-TATA box promoter (e.g. CCAAT) was located to the plus strand. This arrangement predicts a very short 5'-UTR of 17 nucleotides. The significance of the conserved motifs A-E, each with four or more identical nucleotides in sequence, within the promoter regions (Figure 4) is not known.

We have isolated a genomic gene homologous to *NPR1* from *Beta vulgaris*. Functional (proteomic) analyses have been carried out independently by Bargabus-Larson and Jacobsen (2007) with results complimentary to those described here. Since

examples of native gene structure are few for sugarbeet, the present study contributes to the description of the beet genome and further demonstrates that Arabidopsis is a useful model for beet gene discovery and annotation. Remarkable similarity in the exon structure of the *NPR1* orthologs as well as in the intron-exon boundaries in beet and Arabidopsis suggests that the distantly related, but very intensively studied, model plant Arabidopsis shows promise as a predictor of sugarbeet gene function and structure. Further support will come from functional analysis of the *BvNPR1-H1* gene and of the putative control sequences. Moreover, a successful reverse genetic approach could determine if *BvNPR1-H1* has a controlling role in the expression of disease and pest resistance similar to that of the *NPR1* gene in Arabidopsis.

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Figure Legends

Figure 1. Amino acid residue alignment of the complete predicted sugarbeet NPR1 with four eudicot full-length mature predicted protein sequences (GenBank numbers indicated in parentheses). Protein domains are indicated by horizontal shading, and the degree of similarity is indicated by a consensus sequence accompanied by an indication of residue coincidence at individual sites (taller vertical bars indicate higher coincidence among compared accessions).

Figure 2. Phenetic diagram of similarity between predicted sugarbeet NPR1 mature nucleotide sequence and four eudicot NPR1 genes (GenBank accessions are the same as indicated in Figure 1).

Figure 3. Comparison of genomic structures of *BvNPR1-H1* and *Arabidopsis thaliana NPR1*. Polypeptide lengths are indicated above each exon (exons are numbered consecutively from left to right) and nucleotide sequence length is indicated below, highlighting the difference in intron length between these two genes.

Figure 4. Promoter regions of *BvNPR1-H1* and *Arabidopsis thaliana NPR1*. Note the conserved nucleotide sequence boxes A-E and the W-box promoter sites are inverted in beet relative to Arabidopsis. The beet plus strand CCAAT promoter is indicated by "P". Exon 1 includes the 5'-UTR, but is specifically separated here to highlight the predicted 5'-UTR length difference between the two species.

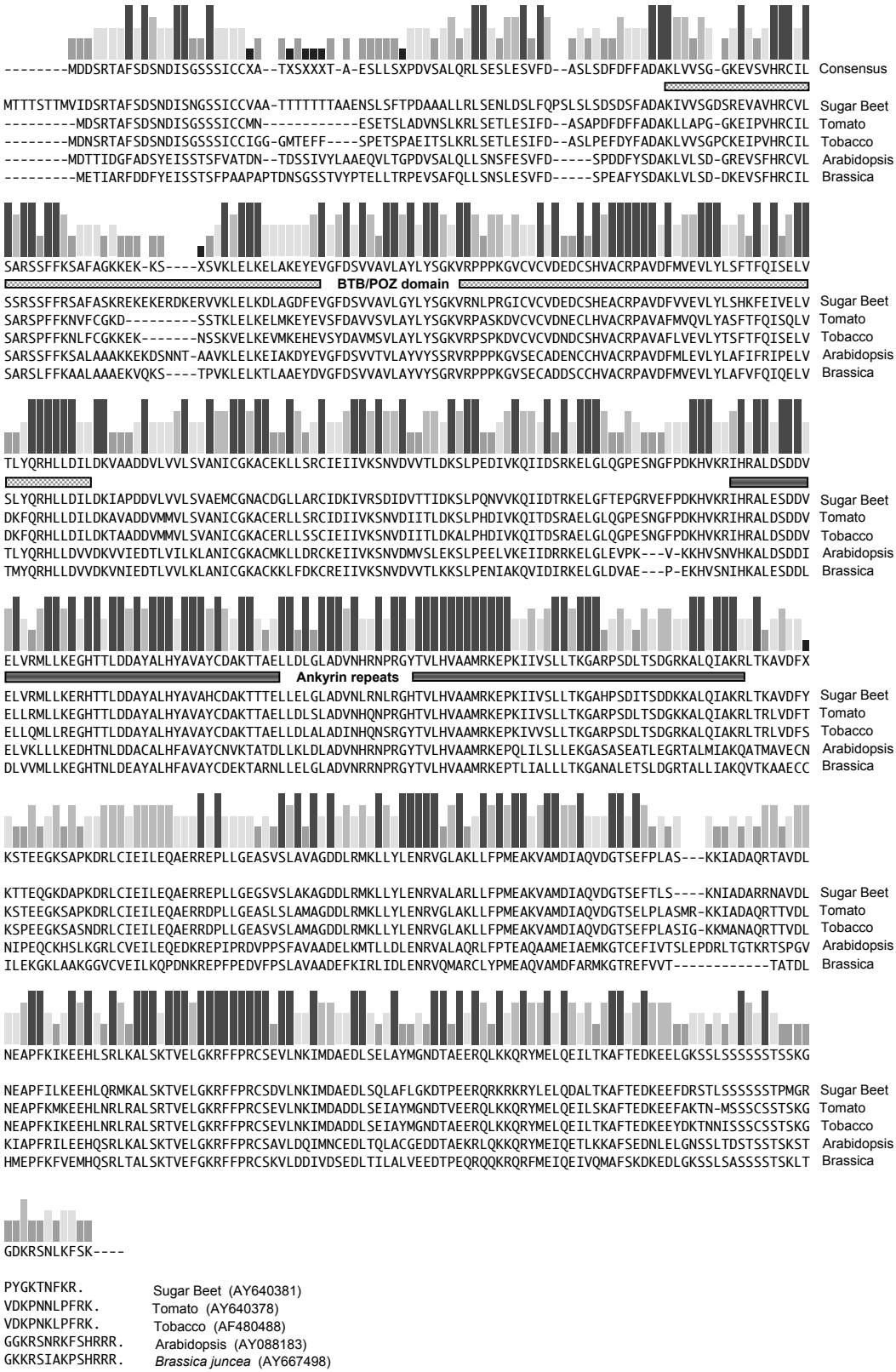


Figure 1: Kuykendall et al.

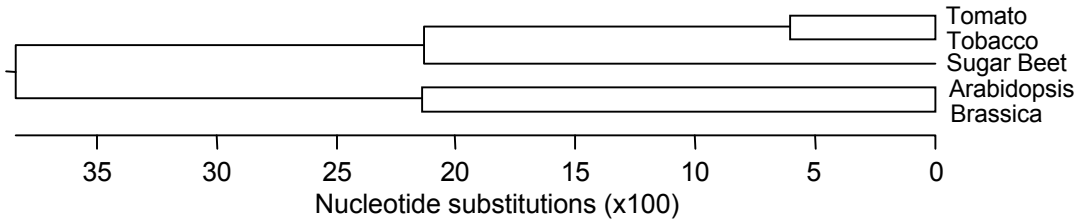


Figure 2: Kuykendall et al.

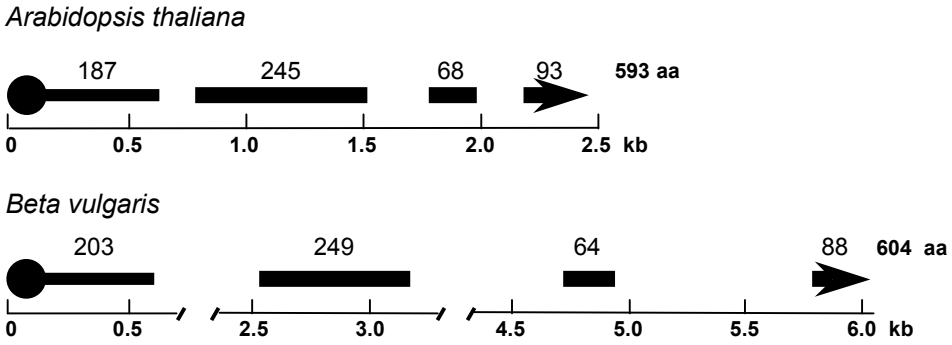
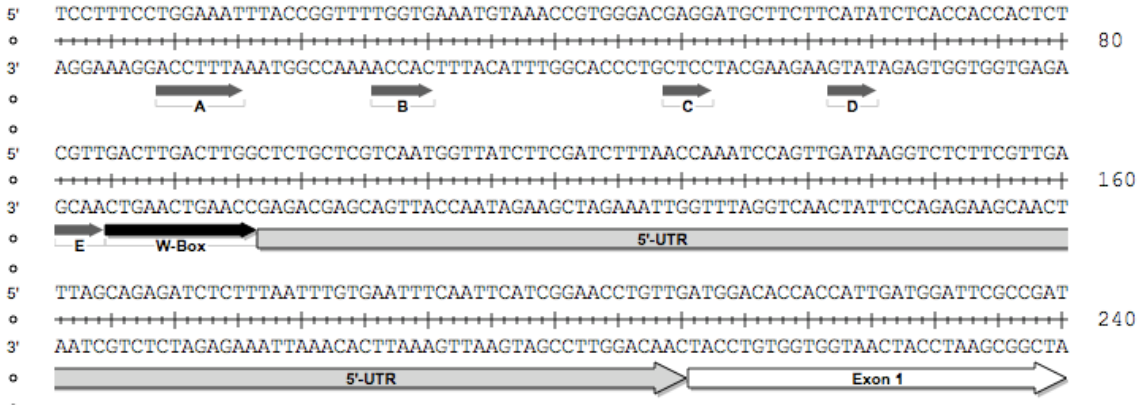


Figure 3: Kuykendall et al.

Arabidopsis thaliana



Beta vulgaris

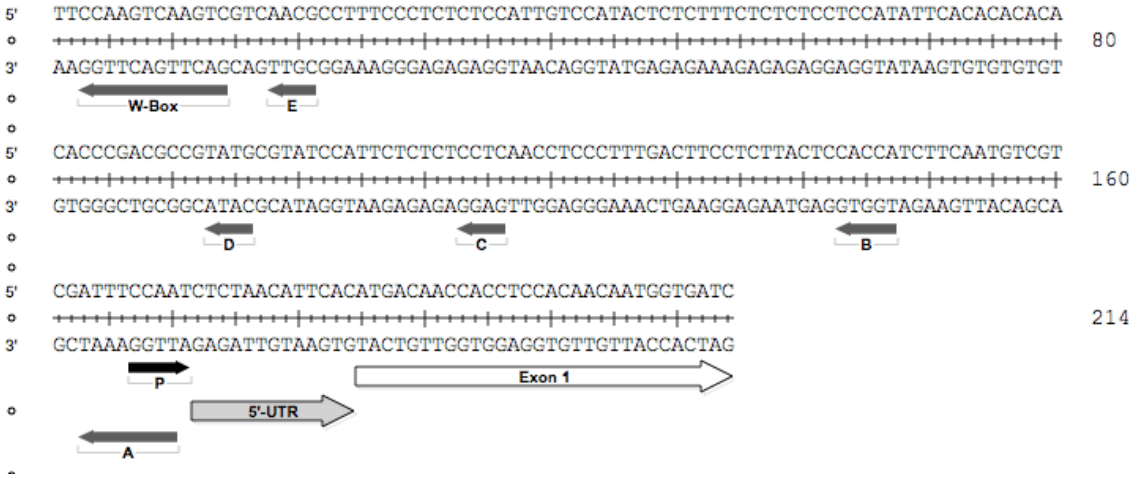


Figure 4: Kuykendall et al.