Brittany Elek and Thomas Pederson

Laboratory Methods in Genomics

Dr. Campbell

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Investigation of Broccoli Quantitative

Trait Loci Genes Associated with Boron

**Abstract**

Broccoli, or *Brassica oleracea*, has many health benefits for humans, as it contains dozens of vitamins and minerals that are important for our bodies (“Broccoli,” 2014). It is important to maintain healthy broccoli so that people can benefit from its nutrients. However, there are boron deficiencies in many countries around the world, including the main producer of broccoli, China (Kato, 2009; “International,” 2012). Boron is an essential nutrient for plants and without it, fruit quality and sets are impaired (Miwa *et al*., 2010). In this study, we found possible genes associated with boron uptake and storage that could be associated with five given quantitative trait loci (QTLs) in broccoli located on chromosomes 3, 4, 6, 8, and 9. We then identified simple sequence repeats (SSRs) in the *Brassica oleracea* genome near the candidate genes. Overall, we were able to find 7 candidate genes (NIP3;1 on chromosomes 6 and 8, and PIP2;3 on chromosomes 3, 4, 6, 8, and 9) for the 5 QTLs as well as 21 SSRs primer pairs for those genes.

**Introduction**

*Brassica oleracea*, or broccoli, is commonly consumed vegetable worldwide. Broccoli, which is rich in many vitamins and minerals, such as vitamin C and vitamin K, is an important source of food for many animals. Furthermore, broccoli has many health benefits for humans, including anti-cancer properties, such as reducing chronic inflammation, oxidative stress, and inadequate detoxification (“Broccoli,” 2014). Although broccoli contains many fundamental vitamins and minerals, these nutrients are unable to benefit humans if there are problems with broccoli production. One major threat to broccoli production around the world is boron deficiency (Kato, 2009). Many regions of the world, such as China and the United States, have limited availability of boron in the soil (Tanaka and Fujiwara, 2008). Furthermore, China and the United States produce almost 63% of the 28,000,000 tons of harvested broccoli each year (“International,” 2012). Therefore it is pertinent for us to maintain healthy broccoli in these areas and understand the ways in which boron affects plants such as broccoli.

Boron deficiency is a problem in many areas across the world, having been reported in at least 80 countries (Kasajima, 2010). Much of the reason for boron deficiency is that 98% of boron is present in the soil as a highly soluble, uncharged molecule boric acid (H3BO3; Tanaka *et al*., 2008). In areas of high rainfall, such as Japan, China, United States, and Brazil, boric acid is easily leached out of the soil, leading to its deficiency (Tanaka and Fujiwara, 2008). The only known function of boron in plants is the cross-linking of pectic polysaccharide rhamnogalacturonan-II, which maintains the cell wall architecture and is necessary for normal growth and development in plants (Kasai *et al.*, 2011). In cases where boron is limited, symptoms mainly occur in growing or expanding organs in the plant rather than mature tissues. These symptoms include inhibition of leaf expansion, root elongation, apical dominance, flower development, and loss of fertility, ultimately leading to decreased fruit and seed sets (Miwa *et al*., 2010).

Although boron deficiency is a major problem worldwide for plant production, boron can also be toxic at high levels, although the molecular mechanism for boron toxicity is still unclear (Kasai *et al*., 2011). At toxic levels, boron causes necrosis of marginal regions of leaves, decreased chlorophyll concentrations, and reduced growth (Miwa *et al*., 2010). Areas where boron toxicity is a problem include, but are not limited to, South Australia, Egypt, and California (Tanaka and Fujiwara, 2008).

One way that we have been dealing with this issue is through the use of fertilizer. However, fertilizer can cause environmental pollution and it is also very costly (Miwa *et al.*, 2006). However, using too much fertilizer could also cause excess boron, which would be toxic to both plants and animals. By examining the broccoli genome and generating boron deficiency-tolerant plants, we may be able to solve these worldwide problems dealing with both boron deficiency and toxicity as well as being able to eliminate the need for as much fertilizer (Kato *et al*., 2009).

It was about 80 years ago when Warington found that boron is an essential nutrient for plants (Miwa *et al*., 2010). However, it was not until recently that scientists have discovered some of the genes associated with boron uptake and storage. There are three mechanisms that are currently known for boron to enter the plant: the first is by passive diffusion, the second by active transport by BOR transporter, and the third is transport by nodulin-like intrinsic protein (NIP) channel (Tanka and Fujiwara, 2008). Since boric acid is an uncharged molecule, it is able to passively diffuse through the lipid bilayer when there is a high supply in the soil (Takano *et al*., 2002). However, when boron is not available in high concentrations, active transport is necessary to import boric acid into the plant. BOR1 was the first gene identified as a boron efflux transporter involved in xylem loading in the plant. It was also found to be necessary for normal shoot growth in low boron concentrations in *Arabidopsis thaliana,* as severe plant growth reduction occurred under boron deficiency when a mutant BOR1 gene was present (Tanka and Fujiwara, 2008). Lastly, facilitated transport by NIP channel was found to also be necessary for normal growth of *Arabidopsis thaliana*.

In one study, two rice genes, OsPIP2;4 and OsPIP2;7 were found to be involved in boron permeability and tolerance. When these genes were overexpressed in *Arabidopsis thaliana*, the plants showed higher biomass production and greater root length (Kumar *et al*., 2013). Both NIPs and PIPs (the rice genes above) are a subset of major intrinsic proteins (MIPs), which function as water-selective or relatively nonselective channels for water and other small, uncharged molecules and are thought to also mediate the transport of boron across the plasma membrane (Takano *et al*., 2006). In *Arabidopsis thaliana*, a total of 35 MIP-encoding genes have been identified. These MIPs are divided into four subfamilies: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), NOD26-like intrinsic proteins (NIPs), and small basic intrinsic proteins (SIPs; Johanson, 2001; Fig. 1).

The goal of our research was to find potential candidate genes that are associated with given QTLs in broccoli. For each candidate gene, we will identify simple sequence repeats (SSRs) so our collaborators can investigate whether or not these genes are actually associated with the given broccoli QTLs.



**Figure 1**. Diagram of the complete set of Major Intrinsic Proteins in *Arabidopsis thaliana*. (Johanson, 2001).

**Methods**

 We thoroughly read literature about boron and its role in *Arabidopsis thaliana*. After reading journal articles, we identified possible uptake genes: BOR1, NIP5;1, and NIP6;1 as well as efflux genes: BOR4 and BOR1. We continued doing research and kept in mind genes that were found in other plants, such as rice. Some of these were NIP2;1, NIP3;1, PIP2;3, PIP2;7, BOR2, and BOT1. After finding candidate genes, we went to the Nation Center for Biotechnology Information (NCBI, 2009) website and found the protein sequences for all of the above genes. We used a tBLASTn and BLASTed the sequences against *B. oleracea*. After BLASTing, we recorded all the hits that had an E-value(expect value) no greater than 1e-06 and were within 10 million base pairs of the QTLs (given in an excel spreadsheet). After using BLAST, we turned to the Integrated Genome Browser (IGB, 2009) in order to search for other potential genes +/- 2 million base pairs away from the QTL of interest. For every gene of interest that we found in IGB (mostly labeled “aquaporin”), we copied the amino acid sequence and BLASTed against the other amino acid sequences of the genes found from BLAST to see if any of the genes showed up in both BLAST and IGB. After finding all of the boron genes that seemed like good candidates for the QTLs, we found three SSR primer pairs that were within +/- 100,000 base pairs of the gene of interest using an excel spreadsheet that we were given by our collaborators.

**Results**

 After investigating 17 possible genes that could be linked to the broccoli QTLs of interest for boron (Fig. 2), we found 7 good candidate genes 5 chromosomes. However, those 7 candidate genes were comprised of only two unique genes: NIP3;1 and PIP2;3 (Figs. 3-7). PIP2;3 was found to be a candidate gene on chromosomes 3, 4, 6, 8, and 9, while NIP3;1 was found to be a candidate gene on chromosomes 6 and 8. From these genes, we were able to find a total of 21 primer pairs (3 for each gene; Fig. 8). Because of how close PIP2;3 and NIP3;1 are located on chromosomes 6 and 8, we were not able to determine which of the genes is a better candidate and they also have the same set of three primer pairs. For a full list of the primer pairs, please see appendix A.



**Figure 2**. Diagram of BLAST results for *B. oleracea* of possible candidate genes within +/- 2 million base pairs of QTLs. Blue boxes represent the chromosomes (with the chromosome number above). Red text represents the QTL, black represents single genes, and blue represents clusters of genes. Numbers below chromosomes indicate the chromosome base-pair length.

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**Figure 3.** See figures 2-6 legend below **Figure 4.** See figures 2-6 legend below.

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**Figure 5.** See figures 2-6 legend below. **Figure 6.** See figures 2-6 legend below.



**Figures 2-6:** IGB vs. BLAST results for chromosomes 3, 4, 6, 8, and 9, in respective order. The QTL is shown in red for each. The IGB results are shown on the left (along with the gene name in the IGB database) and the BLAST results shown on the right (along with the gene name in the BLAST database). The numbers shown are the locations of the gene.

**Figure 7.** See figures 2-6 legend to right.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Gene** | **Chromosome** | **Location Start** | **QTL location** | **No. of Primer Pairs** |
| PIP 2;3\* | 3 | 5,630,235 | 241,916 | 3 |
| PIP 2;3\* | 4 | 4,159,189 | 1,554,267 | 3 |
| NIP 3;1 | 6 | 11,850,945 | 9,847,363 | 3 |
| PIP 2;3 | 6 | 11,851,029 | 9,847,363 | 3 |
| NIP 3;1 | 8 | 32,881,355 | 33,835,534 | 3 |
| PIP 2;3 | 8 | 32,881,978 | 33,835,534 | 3 |
| PIP 2;3 | 9 | 43,534,292 | 41,992,687 | 3+ |

**Figure 8**. Table of potential *B. oleracea* genes associated with Given QTLs on chromosomes 3, 4, 6, 8, and 9. \* denotes that the gene is not located within the 2 million base pair range of the QTL. + indicates that one or more of the primer pairs found surrounded repeats of only two base pairs.

**Discussion**

 Our study has shown that there are five likely candidate genes to be associated with my given QTLs on chromosomes 3, 4, 6, 8, and 9. Although there are five total genes, they are paralogs: PIP2;3 and NIP3;1. It may be likely that the only gene associated with the QTLs are PIP2;3, as this gene is a candidate for the QTL on every chromosome. We are unable to tell whether PIP2;3 or NIP3;1 is a better candidate for the QTLs on chromosomes 6 and 8 because they are so close to each other (BLAST location), and the IGB gene is labeled “aquaporin” and spans the base pairs of both genes according to their BLAST location.

 Although not many candidate genes were found, as there is not much information on the uptake and storage of boron in plants thus far, these genes seem to be great possibilities. IGB labeled the genes that caught our attention as “aquaporin” or “aquaporin-like superfamily protein,” which are also known as MIPs, meaning that it not only allows water, but also small, uncharged molecules such as boric acid (Kumar *et al*., 2013).

 The SSR primer pairs that we found will help to continue the studies about boron and the QTLs of interest in *Brassica oleracea,* thus allowing scientists to alter broccoli in ways that it can be more efficiently grown (such as with less boron), and permitting scientists to selectively breed for the vegetable that requires the least amount of nutrients (if the desired nutrient is scarce). It is crucial that we are able to further understand the genomics of *B. oleracea* so that we can efficiently grow it as well as keep toxins out of the environment from fertilizers that are necessary for normal growth and development at this point in time.

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**Appendix A**: SSR Primer Pairs

**PIP 2;3 Chromosome 3 Location start: 5,630,235**

1)

For primer: TCCACATTAAAGTTCTTGTT

Rev primer: TATTCTCATGGAAAGGTAAA

Repeats: (AAG) x 10 PCR product: 169 Start base: 5,574,061

2)

For primer: ATATTACAATCTGGAAGCAA

Rev Primer: AAGGAAAGAGAGAAGGACTA

Repeats: (TCA) x 8 PCR product: 382 Start base: 5,606,339

3)

For primer: GTAATTCGTTTTTCTTCAAA

Rev primer: AAGGAAAGAGAGAAGGACTA

Repeats: (TCA) x 6 PCR product: 294 Start base: 5,608,487

**PIP 2;3 Chromosome 4 Location start: 4,159,189**

1)

For primer: CTGATCATCTTTTTGTTGAT

Rev primer: TAAGTGTGGCATTTTATTTT

Repeats: (CTT) x 11 PCR product: 364 Start base: 4,123,483

2)

For primer: ATAAGATGGCTTTGTACTCA

Rev primer: TTTTATGAAGGAAAAGAATG

Repeats: (CTT) x 6 PCR product: 255 Start base: 4,169,358

3)

For primer: GTCCTCCACTATAGTTTCCT

Rev primer: GACATAGTGGGTAGATTGAA

Repeats: (TTC) x 5 PCR product: 130 Start base: 4,171,143

**NIP 3;1 Chromosome 6 Location start: 11,850, 945**

1)

For primer: TTAAAAATCAAAATAGCTCC

Rev primer: GAGTTTAAGAAAGCGATGTA

Repeats: (TCTA) x 5 PCR product: 211 Start base: 11,870,200

2)

For primer: TTAATGTTGTGTTTCACGTA

Rev primer: AAAAGAAAAACATGGACATA

Repeats: (TTG) x 6 PCR product: 263 Start base: 11,870,930

3)

For primer: GGGAAAACCTTATTATCTGT

Rev primer: TAATAATCAAAAAGGACCAA

Repeats: (TGT) x 5-(ATT) x 6 PCR Product: 287 Start base: 11,941,798

**PIP 2;3 Chromosome 6 Location start: 11,851, 029**

1)

For primer: TTAAAAATCAAAATAGCTCC

Rev primer: GAGTTTAAGAAAGCGATGTA

Repeats: (TCTA) x 5 PCR product: 211 Start base: 11,870,200

2)

For primer: TTAATGTTGTGTTTCACGTA

Rev primer: AAAAGAAAAACATGGACATA

Repeats: (TTG) x 6 PCR product: 263 Start base: 11,870,930

3)

For primer: GGGAAAACCTTATTATCTGT

Rev primer: TAATAATCAAAAAGGACCAA

Repeats: (TGT) x 5-(ATT) x 6 PCR Product: 287 Start base: 11,941,798

**NIP 3;1 Chromosome 8 Location start: 32,881,355**

1)

For primer: ACGATAAGATAGCAGATTGA

Rev primer: TATTTCGTTCTTTTAATTCC

Repeats: (TTA) x 8 PCR product: 383 Start base: 32,786,764

2)

For primer: ACACATTGAAAATTTGAGAG

Rev primer: AATATAATCATTTGTGGTGG

Repeats: (TAT) x 6 PCR product: 363 Start base: 32,886,988

3)

For primer: GCTGAAAACAGATAAACAAC

Rev primer: ATGTAAGTAGGGATAGGGAC

Repeats: (TCC) x 8 PCR product: 364 Start base: 32,952,627

**PIP 2;3 Chromosome 8 Location start: 32,881,978**

1)

For primer: ACGATAAGATAGCAGATTGA

Rev primer: TATTTCGTTCTTTTAATTCC

Repeats: (TTA) x 8 PCR product: 383 Start base: 32,786,764

2)

For primer: ACACATTGAAAATTTGAGAG

Rev primer: AATATAATCATTTGTGGTGG

Repeats: (TAT) x 6 PCR product: 363 Start base: 32,886,988

3)

For primer: GCTGAAAACAGATAAACAAC

Rev primer: ATGTAAGTAGGGATAGGGAC

Repeats: (TCC) x 8 PCR product: 364 Start base: 32,952,627

**PIP 2;3 Chromosome 9 Location start: 43,534,292**

1)

For primer: TGAATTTATGCTAGTGGATT

Rev primer: AAACAACACATAAGACGAAC

Repeats: (AAG) x 7 PCR product: 334 Start base: 43,460,087

2)

For primer: AGTATAATGTAGCCAACCAA

Rev primer: TTTATCCAATGAAAACAAAT

Repeats: (AT) x 14 PCR product: 344 Start base: 43,469,316

3)

For primer: ATAATATTCGAGGTCCTTTT

Rev primer: TGGATTTTGATTTTGTTTAT

Repeats: (AG) x 16 PCR product: 244 Start base: 43,570,003