Genetic targeting of candidate genes for drought sensitive gene eibi1 of wild barley (Hordeum spontaneum)

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Drought stress is one of the most severe abiotic stresses that cause the loss of crop yield. The cuticle protects the leaf from dehydration in the face of drought stress. The barley cuticle mutant eibi1 is highly drought sensitive. Here, we describe the fine-scale genetic mapping of the eibi1 locus, based on a cv. Morex × eibi1 F2 population of 1,682 individuals. Barley-rice synteny was exploited to identify markers for mapping and to identify candidate genes for Eibi1. The target segment of chromosome 3H is perfectly collinear with the equivalent region on rice chromosome 1. Marker enrichment delimited eibi1 to a 0.11 cM barley region defined by the interval B1958842–Os01g0176800*, which in rice consists of a 112.8 kbp segment. Gene prediction revealed that this rice segment harbours 16 genes. Of them, five (Os01g0177100, Os01g0177200, Os01g0177900, Os01g0178200 and Os01g0178400) were proposed as candidate genes of Eibi1.

Key Words: cuticle, eibi1 mutant, synteny, wild barley, rice, fine mapping.

Introduction

Drought stress is one of most severe agricultural problems affecting plant growth and crop yield (Toenniessen et al. 2003). Many genetic and molecular networks underlying plant adaptation to drought-prone environments have been identified in recent studies (Sreenivasulu et al. 2007), including cuticle metabolism (Kosma and Jenks 2007). The formation of cuticle is a particularly critical plant adaptation to the terrestrial environment (Edwards 1988). Analysis of 1,580 of these has enabled the definition of 79 cer loci, with the wild type allele being dominant at all but one of these. Genetic mapping has placed 27 cer loci, and these appear to be well distributed across six of the seven barley chromosomes (only one cer gene has been mapped to chromosome 6H so far) (Franckowiak 1997). cer genes have yet to be either fine mapped or cloned. The Nad gene in barley shares some homology with the Arabidopsis thaliana WIN1/SHN1 transcription factor gene (Taketa et al. 2008), whose deduced function lies in the control of a lipid biosynthesis pathway. Staining with a lipophilic dye (Sudan black

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B) detects a lipid layer on the pericarp epidermis specifically in covered barley. In covered barley, the contact of the caryopsis surface, overlaid with lipids to the inner side of the hull, generates organ adhesion.

The spontaneous drought-hypersensitive mutant *eibi1* arose in wild barley (*Hordeum spontaneum* Koch) (Chen et al. 2004), and the mutation affects water loss more severely than any other wilty mutants. The *eibi1* gene has a pleiotropic effect on plant morphology, being associated with the development of twisted and dark green leaves, a reduced plant stature, a lower level of fertility, a reduction in spike and seed size, and a slower and lower rate of germination (Chen et al. 2004). The gene has been mapped to the pericentromeric region of bin 6 of chromosome 3H (Chen et al. 2009). We report here the search for candidate genes for *eibi1*, based on high resolution genetic mapping. We have taken advantage of prior knowledge of the identity of flanking markers to focus on a narrow genomic window in a large F2 population bred from the cross cv. Morex × *eibi1*, and have used known syntenic relationships between barley and rice chromosomes to identify the necessary panel of markers mapping in the vicinity of *eibi1*.

**Materials and Methods**

*Plant materials and the leaf drying test*

*H. spontaneum* accession 23–19 is a selection maintained at the Institute of Evolution, University of Haifa, and is the progenitor of the *eibi1* mutant (Chen et al. 2004). Seed of cv. Morex was provided by Professor Andy Kleinhofs, Washington State University, Pullman, USA. An F2 mapping population (1,682 individuals) was derived from the F1 hybrid Morex × *eibi1* (12 plants). Dehusked caryopses of both parental lines and F2 individuals were germinated on filter paper moistened with distilled water and held at 4°C in the dark for five days and at 25°C for a further three days. Seedlings were allowed to continue growing on the filter paper in a covered transparent plastic box. When the first seedling leaf was fully expanded, the distal 2 cm was removed for the leaf-drying test, and an additional 1 cm segment was sampled for DNA extraction. For the leaf-drying test, the leaf explant was placed abaxial side up on tissue paper on a bench under ambient conditions for one hour (Chen et al. 2004). By this time, *eibi1* leaves had become desiccated, but Morex ones remained hydrated (Fig. 1).

**DNA isolation and PCR**

DNA was isolated from seedling leaf samples following Komatsuda *et al.* (1998). The conversion of expressed sequence tags (ESTs) to cleaved amplified polymorphic sequence markers was carried out as described by Pourkheirandish *et al.* (2007). Primers sequences for DNA markers are shown in Table 1. Each PCR contained 20 ng genomic DNA, 300 nM each primer, 200 μM dNTP, 25 mM TAPS (N-tris (hydroxymethyl) methyl-3-amino-propanesulphonic acid, pH 9.3), 50 mM KCl, 1 mM 2-mercaptoethanol, 1.5–4.0 mM MgCl2 and 0.25U ExTaq DNA polymerase (Takara, Tokyo). Each PCR consisted of an initial denaturing step (94°C/5 min), followed by 30 cycles of 94°C/30 s, 55°C to 65°C (primer dependent)/30 s, 72°C/60 s, and completed by

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**Fig. 1.** Drought sensitivity of the *eibi1* mutant. Leaf segments were taken when the first seedling leaf was fully expanded. (A) Morex, *eibi1* mutant, Morex × *eibi1* F1, (B) F2 individuals bred from the cross Morex × *eibi1*. The upper panel shows freshly excised leaf segments, and the lower one the same material after one hour of drying.
an incubation of 72°C/7 min. Amplified DNAs, where necessary following restriction digestion, were electrophoresed through either 1–2% w/v agarose (Iwai Kagaku, Tokyo) gels or, for the separation of fragments smaller than 60 bp, through 3–4% (w/v) Metaphor agarose (Cambrex, Rockland, USA) gels.

Barley–rice synteny and the fine mapping of eibi1

The eibi1 locus lies on chromosome 3H, co-segregating with EST locus AV195814, and is flanked by EST loci BJ474358 and BF263820 (Chen et al. 2009). Barley ESTs predicted to lie in this region were identified by exploiting the established syntetic relationship between this genomic region of barley and the rice genome, and barley orthologues of the resulting rice genes were selected from the Gramene database (http://www.grameine.org/Oryza_sativa/index.html). Multiple copy ESTs were excluded using the TIGR Plant Repeat Database (http://tigrblast.tigr.org/euk-blast/index.cgi?project=plant-repeats), and wheat ESTs were used as a surrogate where no barley ones were available. Gene prediction in rice utilized RAP-DB (Rice Annotation Project 2008) (http://rapdb.dna.affrc.go.jp/cgi-bin/gbrowse/IRGSP40/). Genotyping of the 1,682 Morex × eibi1 F2 individuals at BF263820 and BJ474358 allowed the selection of recombinants within the target genomic region flanking eibi1 and these recombinants were used to identify informative markers in the region. The linkage between candidate linked markers and eibi1 was initially tested on a small sample of F2 individuals, and thereafter on the panel of recombinants. Genetic distances in barley were calculated from the ratio between the number of recombination events and the number of F2 gametes typed.

Table 1. DNA markers used in this study

<table>
<thead>
<tr>
<th>Marker</th>
<th>Primer F (5′-3′)</th>
<th>Primer R (5′-3′)</th>
<th>A.T. (°C)</th>
<th>Restriction enzyme</th>
</tr>
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<tr>
<td>BF263820</td>
<td>GCAGCATTCATCGAGGAGAGAA</td>
<td>AAGCCCTTGTGACGGGAACT</td>
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<tr>
<td>Bmag828*</td>
<td>CATCTTCTAAGGTTTTATCTTTGGA</td>
<td>AGATCTGAGCTCTCAAGAC</td>
<td>60</td>
<td>L.P.</td>
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<tr>
<td>BI958842</td>
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<tr>
<td>BF262108</td>
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<td>TTTCGGCTGTCTTCCTCAACC</td>
<td>60</td>
<td>BstDI</td>
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<tr>
<td>CJ579262</td>
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<td>GCTCCTTATTTCGACCTACCGAT</td>
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<tr>
<td>AV918546</td>
<td>GCAATAGCAGAGGACAGTACG</td>
<td>TCTTTGATGGTCGCCAGGAC</td>
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<tr>
<td>AV833710</td>
<td>CATACCCGGAGAGCAAGCAGTTGT</td>
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<td>AGGACCTGTGTCGAGTTGTG</td>
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<tr>
<td>BJ480900</td>
<td>GGACCAAGCAGACAGCAGTCCG</td>
<td>GCAATAGCAGAGCAGGACAGTTGT</td>
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<td>EcoRV</td>
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<tr>
<td>Os01g0176800*</td>
<td>ACGTGTAGCGCGTACGGTCT</td>
<td>GAGGTGTCGAGCTCTCCTCTCT</td>
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<td>Alul</td>
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<tr>
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<tr>
<td>BE413097</td>
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<td>GGCTGACACATCCCCTCCAAC</td>
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<td>BJ474358</td>
<td>TAAACATCCGCCATCCATCCT</td>
<td>AACCCTATCCAGGCTCCAGGTC</td>
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<td>DdeI</td>
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</tbody>
</table>

Notes:
* Annealing temperature
* Ramsay et al. (2000)
* Length polymorphism, no enzyme

Results

Enrichment of the eibi1 region using barley EST markers

A set of 61 recombinants was identified from the full F2 population of 1,682 individuals. One of these plants carried two different recombinant gametes. The EST AV918546 sequence shares homology with that of rice Os01g0177200 (E-value, 7e−99) on rice chromosome 1 (Fig. 2). A selection of 20 barley ESTs was made on the basis of their presumed orthology with rice genes mapping in the vicinity of Os01g0177200, and the mapping in barley of four of these (BI958842, BJ461432, BJ452857 and CB874399) narrowed the eibi1 window in rice to lie between BI958842* and Os01g0175600. BI958842* represented the rice orthology of barley EST BI958842 since no corresponding locus was annotated for this rice sequence. A second round of barley EST selection targeted this rice interval, producing 21 candidates, of which five (BF262522, AV833710, BJ480900, BF262108 and BE413097) were mapped. This process redefined the position of the rice Eibi1 orthologue to the interval between BI958842* and Os01g0176800 (Fig. 2).

Marker development using wheat ESTs and rice genomic sequence

The sequences of the wheat ESTs CJ579262, CD887967 and CD902914 share homology with the rice sequences Os01g0177900, Os01g0177200 and Os01g0176500, respectively. CJ579262 and CD887967 co-segregated with eibi1 while CD902914 co-segregated with BF262108 (Fig. 2). The Os01g0177200 sequence shares homology with that of three ESTs (AV918546, AV833710 and CD887967). No barley or wheat homologues of Os01g0176800 were identifiable from pre-existing sequence, so its putative open...
A sequence polymorphism between the Morex and *eibi1* amplicons was used to show that the locus (designated Os01g0176800* since its marker was derived from rice Os01g0176800) co-segregated with BF262108 and CD902914 (Fig. 2). The interval BI958842 to Os01g0176800* limited the *eibi1* segment to be of genetic length 0.11 cM. In rice, the physical length of the syntenous segment (BI958842 to Os01g0176800) is 112.8 kbp (Fig. 2).

**Candidate genes for Eibi1**

A comparison between the barley genetic map and the rice physical map revealed that the barley region BI958842–
UBP14 is associated with an embryo-lethal phenotype by the 26S proteasome. In released from proteins during or following their breakdown ubiquitins via the disassembly of free multi-ubiquitin chains protease 14. This enzyme is responsible for the recycling of possible candidate for 2006). Its negative effect on fertility makes this gene a plausible candidate for patterning (Liu 1997).

Discussion

Candidate genes for Eibi1

Among the 16 genes located in the appropriate genomic region, five represented the most likely candidates for Eibi1. These were Os01g0177100, Os01g0177200, Os01g0177900, Os01g0178200 and Os01g0178400. Os01g0177100 encodes a product similar to STYLOSA (STY) in Antirrhinum majus and LEUNIG (LUG) in A. thaliana. These genes play a key regulatory role in cell fate specification, hormone signalling, and plant stress responses. leunig mutants are defective in their gynoecium development, show reduced female and male fertility, distorted leaf and floral organ shape, and non wild-type vascular patterning (Liu et al. 2000, Cnops et al. 2004, Franks et al. 2006). Its negative effect on fertility makes this gene a plausible candidate for Eibi1.

Os01g0177200 is predicted to encode ubiquitin-specific protease 14. This enzyme is responsible for the recycling of ubiquitins via the disassembly of free multi-ubiquitin chains released from proteins during or following their breakdown by the 26S proteasome. In A. thaliana, the absence of UBP14 is associated with an embryo-lethal phenotype (Doelling et al. 2001, Tzafrir et al. 2002), whereas yeast ubp14Δ mutants are only mildly compromised (Amerik et al. 1997).

The Os01g0177900 gene product is an ABC-2 type transporter domain containing protein, predicted to participate in the transport of secondary metabolites. It is involved in various plant-pathogen interactions, and may also contribute to the transport of signalling molecules and the secretion of volatiles. Factors which promote the expression of plant ABC-2 type transporters include cycloheximide, brassinolides, herbicides, heavy metals, jasmonates, auxins, cytokinins and a wide range of microbial elicitors and stress factors (including cold and salinity) (Rea 2007). The Eibi1 product could be an ABC-2 type transporter, since a defect in the transport of secondary metabolites from the epidermis to the cuticle could explain the heightened cuticle permeability characteristic of the eibi1 mutant (Chen et al. 2004). In A. thaliana, the knockout mutation of AtWBC11 (an ABC transporter gene) produced organ fusions and altered cuticle permeability (Bird et al. 2007, Luo et al. 2007).

The predicted product of Os01g0178200 is a transmembrane family protein, bridging the internal and external surfaces of the membrane or lipid bilayer in which it is embedded. It mediates a wide range of processes, including cell signalling (Bennasroune et al. 2004), the transport of membrane-impermeable molecules (Bird et al. 2007) and cell to cell communication (Hake and Char 1997). The rationale for considering Os01g0178200 to be a candidate for Eibi1 is that transmembrane proteins are presumably involved in the transport of lipids to the developing cuticle.

Finally, Os01g0178400 is predicted to encode a protein phosphatase 2A (PP2A) regulatory B subunit containing protein. The PP2As are an abundant class of oligomeric enzymes, which play an important role in the regulation of growth and development. Altered PP2A activity in plants has been associated with defects in hormone homeostasis and signalling, defence responses, cell division, morphogenesis, and reproduction (DeLong 2006).

Os01g0178000 (a predicted aminotransferase) and Os01g0177400 (a predicted gibberellin 3 oxidase gene) are the two remaining functionally assigned genes in the target rice segment. Neither of these is likely to be a candidate for Eibi1, since there is no discernible connection between their presumed function and cuticle development. Aminotransferases play a role in nitrogen and carbon metabolism, particularly in legume root nodules and the leaves of C₄ species. Its functions include the catabolism and biosynthesis of aspartate, the conversion of tricarboxylic acid cycle intermediates to amino acids, the assimilation of fixed nitrogen into asparagine in the nodules, and the redistribution of nitrogen and carbon pools between the cytoplasm and other compartments (Silvente et al. 2003). Gibberellin 3 oxides catalyse a late step in the synthesis of active gibberellic acid. In rice, the d8 mutant is severely dwarfed, but neither flower nor grain development are compromised. The gene is important for the control of bioactive gibberellin synthesis required for shoot elongation, but not for reproductive development (Sakamoto et al. 2003). There is no known connection between gibberellin synthesis and cuticle development.
A further seven hypothetical protein encoding genes and two conserved hypothetical protein encoding genes are present in the critical rice genomic region (Table 2). A full candidate gene analysis is difficult to achieve because the number of candidates is too large. One way of reducing this number would be increase the size of the mapping population in order to improve the fine-scale resolution of the genetic map. However, this approach would fail if no orthologue of Eibi1 were present in rice as the case of six-rowed spike gene vrs1 (Pourkheirandish et al. 2007). In this case, the two best current alternative approaches would be either to generate a localised BAC contig in barley and analyse its gene content (Komatsuda et al. 2007), and/or to resort to the use of the model temperate grass Brachypodium distachyon as a replacement for rice (Sakuma et al. 2009).

Eibi—a possible new cuticle gene

Genes involved in the development of the cuticle have been characterized in Arabidopsis, maize, and sorghum, but none of rice orthologues of these map in the eibi region in rice genome. Such genes include LACS2 (Schnurr et al. 2004) on chromosome 11, WD40 (Jung et al. 2006), AtWBC11 (Bird et al. 2007, Luo et al. 2007, Panikashvili et al. 2007, Ukitu et al. 2007), CER1 (Aarts et al. 1995) and BODYGUARD (Kurdyukov et al. 2006) on chromosome 10, WX2/YRE/FLP1/CER3 (Rowland et al. 2007) and GLOS51 (Sturaro et al. 2005) on chromosome 9, WSD1 (Samuels et al. 2008) and CER4 (Rowland et al. 2006) on chromosome 8, GLOSSY15 (Moos and Sisco 1994) on chromosome 6, FIDDLEHEAD (Yephremov et al. 1999) and CER5/AtWBC12 (Pighin et al. 2004) on chromosome 5, GLOSSY8 (Xu et al. 1997), GLOSSY12 (Tacke et al. 1995) and CER2 (Xia et al. 1997) on chromosome 4, CUT4/CER6 (Fiebig et al. 2000) and KCS1 (Todd et al. 1999) on chromosome 3, WIN1 (Broun et al. 2004), SHN1 (Aharoni et al. 2004), CER7 (Hoeker et al. 2006), FATB (Bonaventure et al. 2003) and ATT1 (Xiao et al. 2004) on chromosome 2. Both CER10 (Zheng et al. 2005) and RESURRECTION1 (Chen et al. 2005) map to chromosome 1, but neither is located within the eibi window. Given the perfect collinearity between rice and barley in the eibi segment, as already demonstrated for barley chromosome 3H and rice chromosome 1 (Stein et al. 2007), it is highly likely that a rice orthologue of Eibi1 is indeed present. If so, this would represent an as yet undescribed rice gene involved in cuticle development.

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Literature Cited


Fiebig, A., J.A. Mayfield, N.L. Miley, S. Chau, R.L. Fischer and D. Preuss (2000) Alterations in CER6, a gene identical to CUT1, differentially affect long-chain lipid content on the surface of


