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# Mapping of the *eibi1* gene responsible for the drought hypersensitive cuticle in wild barley (*Hordeum spontaneum*)

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Segregation analysis showed that *eibi1*, a drought hypersensitive cuticle wild barley mutant, was monogenic and recessive, and mapped in two $F_2$ populations, one made from a cross between the mutant and a cultivated barley (cv. Morex), and the other between the mutant and another wild barley. A microsatellite marker screen showed that the gene was located on barley chromosome 3H, and a set of markers already assigned to this chromosome, including both microsatellites and ESTs, was used to construct a genetic map. *eibi1* co-segregated with barley EST AV918546, and was located to bin 6. The synteny between barley and rice in this region is incomplete, with a large discrepancy in map distances, and the presence of multiple inversions.

**Key Words:** wild barley, *Hordeum spontaneum*, *eibi1* mutant, genetic mapping, rice, synteny.

## Introduction

A waxy cuticle covers the aerial surfaces of the leaf and stem of many plants. The cuticle consists of two layers, the outer, translucent cuticle proper and an inner, opaque cuticular layer (Jeffree 1996). One of the cuticle's most important functions is to control water loss, a property which is especially important for plants growing in drought-prone environments (Riederer and Schreiber 2001, Jenks *et al.* 2002). The imposition of drought stress can affect both the thickness and the composition of the cuticular wax formed (Shepherd and Griffiths 2006). Current understanding of how this process is controlled is emerging from studies of cuticle mutants such as *eceriferum* (*cer*) in *Arabidopsis thaliana* and barley, and *glossy* (*gl*) in maize and various Brassicaceae; in these mutants, the synthesis of cuticular wax is blocked at various points, leading to the accumulation of intermediates (Jenks *et al.* 2002).

An increase in cuticle permeability has been associated both with a decrease (Jenks *et al*. 1994, Sturaro *et al.* 2005) and an increase in cuticular thickness (Chen *et al.* 2003), a change in cuticle polymer type (Xiao *et al.* 2004) or in the composition of the wax (Aharoni *et al.* 2004, Vogg *et al.* 2004). The metabolism responsible for the formation of the cuticle appears to be finely tuned, with only limited plasticity available to the plant to compensate for genetic changes. This may explain why such a large number of different cuticle mutants have been identified.

At least 21 genes involved in cuticle formation have been isolated to date. Of these, seven encode regulatory loci (*CER2*, *CER7*, *GL15*, *RESURRECTION1*, *SHIN*, *WAX2*/*YRE*/*FLP1*/*CER3*, *WIN1*), ten encode enzymes (*ATT1*, *BODYGUARD*, *CER4*, *CER6*/*CUT1*, *CER10*, *FIDDLEHEAD*, *GL2*, *GL8*, *KCS1*, *LACS2*) and the remaining four (*CER1*, *CER5*, *GL1*, *AtWBC11*) are thought to be involved in the transport of wax compounds (Bird *et al.* 2007, Jenks *et al*. 1995, Kunst and Samuels 2003, Pighin *et al.* 2004, Rowland *et al.* 2007, Shepherd and Griffiths 2006). In barley, 1,890 *cer* mutants have been identified following mutagenesis (Lundqvist and Lundqvist 1988), and the genetic basis of 1,580 of these has been assigned to 79 *cer* loci, all but one of which act as recessive genes. A total of 27 distinct *cer* loci are distributed over all seven barley chromosomes, although only one maps to chromosome 6H (Franckowiak 1997). None of the barley *cer* genes has yet been either fine mapped or cloned.

The spontaneous *eibi1* mutant (named after Eibi, Eviator Nevo) was identified in wild barley (*Hordeum spontaneum* Koch), and is hypersensitive to drought (Chen *et al.* 2004). Its relative water loss rate is higher than that of other wilty mutants. Compared to the wild type, treatment of *eibi1* leaves with 80% ethanol results in a five fold higher rate of chlorophyll efflux. The high transpiration rate of *eibi1* is not related to an abnormal stomatal density. The defective *eibi1* cuticle not only enhances the plant's sensitivity to drought stress, but also is associated with the rapid loss of water from

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detached leaves, a twisted and dark green leaf phenotype, shorter plant stature, a twisted peduncle, a high tiller number, reduced fertility, smaller spikes, smaller grains, delayed germination, and a reduced germination rate (Chen *et al.* 2004).

We report here the genetic mapping of *eibi1*, achieved by applying microsatellite assays to segregating populations. We have taken advantage of the established synteny between barley and rice to provide sufficient molecular markers in the *eibi1* region to identify a locus that co-segregates with *eibi1*.

## Materials and Methods

*Plant materials*

Two wild barley accessions (23-19 and 23-73) were selected from the Wadi Qilt population maintained at the Gene Bank of the Institute of Evolution, University of Haifa, Israel. The *eibi1* mutant arose from 23-19 (Chen *et al.* 2004). Seed of the barley cultivar Morex was obtained from Washington State University, Pullman, USA. Dehusked caryopses of these lines were germinated on wetted filter paper and kept at 4°C in the dark for 5d, and then at 25°C for 3d. The seedlings were then transplanted to 2.5 l soil-filled pots, and grown to maturity in a greenhouse. Crosses were made between *eibi1* (as male) and either Morex, 23-19 or 23-73, and the resulting $F_1$ plants used to derive three independent $F_2$ populations. $F_1$ and $F_2$ seedlings were allowed to grow for an additional 3d beyond the normal germination time in Petri dishes in a transparent box, to obtain the material required for a leaf-drying test. Thereafter, the seedlings were grown in a greenhouse as normal. The leaf-drying test used a ~2 cm long leaf segment taken from the tip of the first expanded seedling leaf. This was held abaxial side up on tissue paper at room temperature for 1 h, following the procedure described by Chen *et al.* (2004).

*DNA isolation, PCR conditions, and genetic map construction*

DNA was extracted from fresh seedling leaf following Komatsuda *et al.* (1998). Each PCR mixture (10 µl) contained 20 ng genomic DNA, 300 nM of 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 (primer dependent) $MgCl_2$ and 0.25U ExTaq DNA polymerase (Takara, Tokyo). The PCR began with an incubation at 94°C for 5 min, followed by 30 cycles of 94°C/30 s, 55°C to 60°C (primer dependent)/30 s and 72°C/60 s, and ended with an incubation for 7 min at 72°C. Amplicons were electrophoresed through 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. Linkage maps were constructed using MAPMAKER v3.0 software (Lincoln *et al.* 1993). Kosambi's mapping function (Kosambi 1944) was used to convert recombination frequencies into map distances.

*Molecular marker selection*

A set of genome-wide SSR markers (Ramsay *et al.* 2000) was applied to a sub-sample of 43 mutant phenotype $F_2$ segregants from another Morex × *eibi1* population to establish the chromosomal location of *eibi1*. Then, SSR assays defining loci on the candidate chromosome (3H) were used to map *eibi1* in other $F_2$ populations derived from both Morex × *eibi1* (155 individuals) and 23-73 × *eibi1* (108 individuals). These $F_2$ populations were different from the $F_2$ populations used in segregation analysis (Table 1) and the chromosomal location of *eibi1* (Table 2). Eleven CAPS markers found in the Haruna Nijo × OUH602 map (K. Sato, unpublished) were developed and used for further genotyping of the Morex × *eibi1*, 23-73 × *eibi1* and Azumamugi (AZ) × Kanto Nakate Gold (KNG) (Komatsuda and Mano 2002). Twenty barley ESTs, predicted as mapping close to AV922032 (a barley EST located on chromosome 3H; N. Senthil and T. Komatsuda, unpublished) on the basis of synteny with rice chromosome 1, were also used for mapping.

*Rice* in silico *map*

The rice homologue of each barley EST was identified by BLAST analyses against sequences deposited in the NCBI (http://www.ncbi.nlm.nih.gov), Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/), and Rice Annotation Project (RAP, http://rapdb.dna.affrc.go.jp/). Barley ESTs homologous with predicted rice genes were searched in GRAMENE (http://www.gramene.org/) and RAP-DB (http://rapdb.dna.affrc.go.jp/) using "G-integra" function. MapChart software (Voorrips 2002) was used to draw both the rice physical map and the barley genetic maps.

**Table 1.** Segregation of plants with the *eibi1* phenotype in the $F_2$

| Crosses | Wild type | Mutant type | $\chi^2$ for 3 : 1 |
|---|---|---|---|
| Morex × *eibi1* | 117 | 41 | 0.076 |
| 23-19 × *eibi1* | 157 | 55 | 0.101 |
| 23-73 × *eibi1* | 159 | 60 | 0.671 |

**Table 2.** Linkage between *eibi1* and SSRs in a $F_2$ sub-population of individuals showing the mutant *eibi1* phenotype of the cross between Morex (A) and *eibi1* (B). The population was different from the Morex × *eibi1* population of Table 1.

| Chromosome | SSR | Genotype | | | $\chi^2$ for 1 : 2 : 1 |
|---|---|---|---|---|---|
| | | BB | AB | AA | |
| 1H | Bmag579 | 11 | 21 | 10 | 0.05 |
| 2H | Hvm36 | 11 | 25 | 6 | 2.71 |
| 3H | Hvm60 | 24 | 15 | 4 | 22.53*** |
| 3H | Bmag603 | 38 | 4 | 0 | 96.29*** |
| 4H | Hvm67 | 11 | 17 | 14 | 1.95 |
| 5H | Hvm68 | 12 | 18 | 11 | 0.66 |
| 6H | EBmac684 | 10 | 21 | 12 | 0.21 |
| 7H | EBmag757 | 12 | 23 | 6 | 2.37 |

*** Significant at the 0.1% probability level.

## Results

*The* eibi1 *is a single recessive nuclear mutation*

While wild-type seedlings at the five-leaf stage wilted after water was withheld for 5d, *eibi1* seedlings had wilted within 3d. After re-watering, the leaf tips of *eibi1* seedlings remained wilted, but those of the wild type recovered (Fig. 1A). The genotypes differed substantially in response to the leaf-drying test (Fig. 1B), with *eibi1* leaf segments losing 43.2% of their initial fresh weight within 1 h, and the wild-type only 4.6%. The appearance of the former leaf segments was dry and shrunken, but that of the latter were still fresh, and this phenotype proved reliable for scoring the $F_2$ segregants. In each of the three mapping populations, the trait segregated according the Mendelian expectation of a monogenic recessive gene (Table 1), indicating that the *eibi1* mutation was caused by a single recessive nuclear mutation.

*The* eibi1 *gene is located on chromosome 3H*

Of the 40 SSR markers used for the genome-wide screen, 34 were informative between *eibi1* mutant and Morex. When one SSR per chromosome was used to genotype the 43 $F_2$ plants with mutant phenotype of the Morex × *eibi1* population, which were selected from a population different from the population in Table 1, only Hvm60 and Bmag603 (chromosome 3H) showed a linkage with *eibi1* (Table 2). In the full Morex × *eibi1* $F_2$ population, Bmag603 and Bmag828 were both closely linked with *eibi1* (6.2 cM and 1.0 cM, respectively) (Fig. 1C). In the 23-73 × *eibi1* $F_2$ population, the Bmag603–*eibi1* distance was 7.7 cM.

Of the barley ESTs from Haruna Nijo × OUH602 map to this region of chromosome 3H, four were mappable in Morex × *eibi1*, and five in 23-73 × *eibi1* (Fig. 1C, blue letters). Barley EST AV922032 (green letters in Fig. 1C) co-segregated with *eibi1* in 23-73 × *eibi1*, but was non-informative in the Morex × *eibi1* population (Fig. 1C). As a rice homologue (Os01g0188100) of barley AV922032 is located on rice chromosome 1, synteny was used between this region and barley chromosome 3H to identify a further 20 barley ESTs as potential markers for *eibi1*. Four and three of the 20 ESTs were mappable in, respectively, the Morex × *eibi1* and 23-73 × *eibi1* populations (Fig. 1C, red letters). In the 23-73 × *eibi1* populations, all three co-segregated with *eibi1*, while in the Morex × *eibi1* populations, the four loci delimited a 6.5 cM region spanning *eibi1*. EST AV918546 co-segregated with *eibi1* was found newly in both populations (Fig. 1C).

## Discussion

*SSR marker assisted identification of the chromosomal location of* eibi1

A procedure which allows the rapid assignment of mutant alleles to linkage group was described by Castiglioni *et al.* (1998), and was applied to a set of homozygous recessive $F_2$ segregants to map *branched*, *calcaroides*, and 29 other developmental mutants (Castiglioni *et al.* 1998, Pozzi *et al.* 2000, Pozzi *et al.* 2003). The same approach was taken by Pratchett and Laurie (1994) to locate the recessive liguleless locus to chromosome 2H, based on the genotyping of just 36 $F_2$ segregants. Once the monogenic nature of the *eibi1* had been established by segregation analysis, a genome-wide SSR scan of a small subset of $F_2$ plants was sufficient to identify the likely chromosomal location of the gene responsible for the mutant phenotype. The linkage of *eibi1* with Hvm60 indicated by the coarse-resolution scan suggested that *eibi1* was located on chromosome 3H, and this location was confirmed by full population mapping using other SSRs and EST loci known to map to this chromosome or chromosome region.

eibi1 *maps to the pericentromeric region of chromosome 3H*

Bmag828 and Bmag603 flanked *eibi1* in the Morex × *eibi1* map (Fig. 1C). The former locus maps in position 92.1 cM close to the centromere of chromosome 3H in the Steptoe × Morex SSR map (Li *et al.* 2003). Bmag603 maps to 43.5 cM close to the centromere on chromosome 3H in the 2003 barley consensus map (Karakousis *et al.* 2003). This pericentromeric region contains at least eight barley ESTs in the region between 37.8 cM and 58.3 cM of Haruna Nijo × OUH602 map (K. Sato, unpublished data), and some of these are linked to *eibi1* (Fig. 1C).

*Synteny between rice and barley in the chromosome 3H pericentromeric region*

Comparative mapping has revealed extensive conservation of gene content and order among the genomes of cereal crops (Devos and Gale 2000). Synteny between barley chromosome 3H and rice chromosome 1 is well documented, with gene order largely maintained (Smilde *et al.* 2001). Over 69% (91 out of 131) of barley ESTs on chromosome 3H are homologous to the coding sequences of the corresponding genes on rice chromosome 1 (Stein *et al*. 2007). A similar proportion of unigene sequences (34 out of 50, 68.0%) on barley chromosome 3H matches the unigenes on rice chromosome 1 (Rostoks *et al*. 2005). These proportions were maintained in the present study, where seven out of ten barley ESTs were well matched with sequences on rice chromosome 1. However, collinearity was disturbed within the syntenic region defined by these ten barley ESTs, and genetic distances were also discrepant (Fig. 1C). The genetic distance between CB859971 and AV928420 was 19.9 cM in the Morex × *eibi1* map (Fig. 1C), whereas the genetic distance of this region (Os01g0193500–Os01g0719100, Fig. 1C) in rice spans 97.2 cM (http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice, "Rice Genetic Markers"). Smilde *et al.* (2001) observed a 30-fold suppression of recombination in barley chromosome 3H pericentromeric region compared to rice. Han *et al.* (1998) estimated a 6- to 15-fold suppression in recombination rate in the pericentromeric region of barley chromosome 7H compared to rice orthologous region. The different degrees of suppression reported in the literature


C

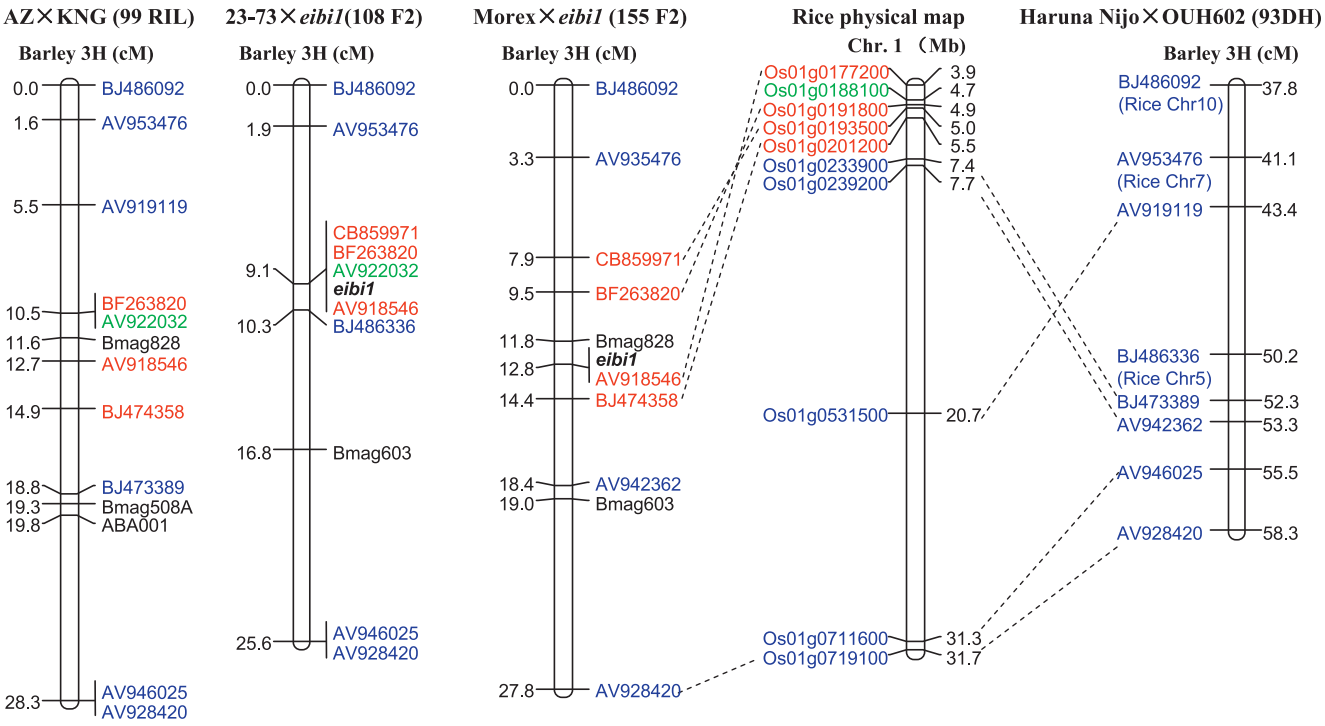


**Fig. 1.** The drought sensitivity of the *eibi1* mutant. (A) Five-leaf stage seedlings rehydrated after a drought stress applied by withholding water for 5d. Left hand pair of plants are *eibi1*, and the right hand pair are wild type. (B) Leaf segments cut from *eibi1* (above) and wild type (below) plants after drying for 1h. (C) Genetic maps of the *eibi1* gene in barley chromosome 3H and the syntenic region of rice chromosome 1. The barley EST map was adapted from Haruna Nijo×OUH602 map (blue letters) and the conserved order of markers was shown in four barley mapping population. AV922032 (green letter) was a barley EST located on chromosome 3H (N. Senthil and T. Komatsuda, unpublished). EST markers in red were developed based on barley–rice synteny. Dashed lines join barley ESTs with their rice homologues. Genetic distance of barley is shown in centimorgans (cM) and the physical map distance of rice is shown in mega-bases (Mb). These $F_2$ populations were different from the $F_2$ populations used in segregation analysis (Table 1) and the chromosomal location of *eibi1* (Table 2).

may merely reflect chromosome-to-chromosome or chromosome region-to region variation. The genetic background can also affect estimates of map distance, as for example the CB859971 to AV918546 interval which spanned 4.9 cM in the Morex × *eibi1* map, but 0 cM in the 23-73 × *eibi1* map. This may suggest that the fine mapping of *eibi1* would be

more effective in the former cross.

Barley chromosome 3H and rice chromosome 1 have a collinear organization over almost their entire length (Rostoks *et al.* 2005, Smilde *et al*. 2001, Stein *et al*. 2007). However, we have observed in this study that the pericentromeric syntenic region is disrupted by two inverted blocks and one transposition (Fig. 1C). Transpositions are particularly problematical for synteny-based cloning. In the case of map-based cloning of the *Vrs1* gene, extensive gene sequence conservation existed between barley chromosome 2H and rice chromosome 4 (Pourkheirandish *et al*. 2007), allowing 22 EST-based STS markers to be placed within the critical region, and their linear order was identical in barley and rice. However, the rice *Vrs1* homologue mapped to rice chromosome 7, presumably as a result of a transposition. Such breakdowns in micro-collinearity emphasize the limitations of synteny-based cloning, and stress the importance of implementing genomic studies directly in the target species (Pourkheirandish *et al.* 2007).

*Is* eibi1 *a new barley cuticle gene?*

The *eibi1* cuticle is defective, raising the possibility that it represents a new cuticle gene, alongside the 27 *cer* genes distributed throughout the genome (Franckowiak 1997). Four *cer* genes (*cer-zd*, *cer-r*, *cer-zn*, and *cer-b* (*gsh2*)) map within, respectively, bins 6, 7, 8, and 10 of chromosome 3H. Bin 6 also contains *eibi1* because the interval defined by the two RFLP loci ABG462 and MWG2015 includes both Bmag828 and Bmag603—which flank *eibi1*—and both RFLP loci map to bin 6. However, since *cer-zd* lies outside the ABG462–MWG2015 interval, *eibi1* and *cer-zd* cannot be the same locus. Thus, as *eibi1* is not one of the previously identified mapped *cer* genes, it appears to be a new barley cuticle gene.

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